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**ASPECTS OF THE PHARMACOKINETICS
AND PHARMACODYNAMICS OF
CHLORAMPHENICOL, ENROFLOXACIN
AND FLUCONAZOLE IN KOALAS
(*PHASCOLARCTOS CINEREUS*)**

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**A thesis submitted in fulfilment of the requirements for
the degree of Doctor of Philosophy**

Faculty of Veterinary Science

The University of Sydney

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STATEMENT OF ORIGINALITY

Apart from the assistance that has been acknowledged, this thesis represents the unaided work of the author. The text of this thesis contains no material that has been previously published or written unless due reference to this material has been made. This work has neither been presented, nor is currently being presented, for any other degree.

Lisa Black

December 2013

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SUMMARY OF THE THESIS

In Australia, thousands of koalas are presented to wildlife rehabilitation facilities annually for medical treatment. Most admissions are due to trauma (dog attacks and motor vehicle strikes) and disease. The most common infectious disease treated in koala rehabilitation facilities is chlamydiosis, caused by the intracellular bacteria, *Chlamydia pecorum* and *Chlamydia pneumoniae*. Although less common, the fungal disease cryptococcosis, caused by *Cryptococcus gattii*, also occurs and is often fatal. Despite the large number of koalas administered therapeutic drugs in Australia annually, there is a paucity of information regarding the pharmacokinetics and pharmacodynamics of commonly used therapeutic drugs in this species. The information available indicates koalas may have highly developed barriers to oral absorption of therapeutic drugs, and an ability to rapidly metabolise and eliminate therapeutic drugs following administration; these mechanisms are likely to have evolved due to the highly toxic *Eucalyptus* spp. diet that these specialist folivores ingest. This thesis aims to describe the pharmacokinetics of three drugs commonly administered to koalas, chloramphenicol, enrofloxacin and fluconazole, and to use pharmacokinetic/pharmacodynamic (PK/PD) principles to draw conclusions regarding the theoretical efficacy of these drugs when administered at commonly used dosages.

The literature review presented in Chapter 1 outlines the history and conservation status of the koala in Australia, and discusses the two most common infectious diseases of wild koalas, chlamydiosis and cryptococcosis. Literature regarding the medical management of these diseases is considered, current treatment recommendations

discussed, published case studies reviewed, and gaps in the current knowledge of drug pharmacokinetics in koalas identified.

Pharmacokinetic studies of chloramphenicol and enrofloxacin, both commonly used to treat chlamydiosis in koalas, are presented in Chapters 2 and 3. The elimination rate of both drugs is found to be similar to other mammals, and consideration of the pharmacodynamics reveals that chloramphenicol is likely to be a suitable treatment option for koala chlamydiosis, although slight dosage adjustments may be necessary. Conversely, enrofloxacin is found to be unsuitable for treating chlamydiosis in koalas. Enrofloxacin may, however, show promise for treating infections caused by gram positive bacteria with low minimum inhibitory concentrations (MICs).

Chapter 4 investigates the pharmacokinetics of fluconazole, commonly used to treat cryptococcosis. Pertinent findings include poor and variable oral absorption, rapid elimination, and absorption rate-limited disposition following oral administration; these findings are in stark contrast to the pharmacokinetics of fluconazole in other species that have been studied. Consideration of the pharmacodynamics indicates that fluconazole is not likely to be successful in treating cryptococcosis at currently used dosages when used alone. Interestingly, this study demonstrates that fluconazole is unsuitable for allometric scaling in koalas, despite demonstrating a strong allometric relationship in other mammalian species ranging in size from mice to whales.

Chapter 5 was born out of a need to determine the *in vitro* susceptibility of koala isolates of *Chlamydia pecorum* to enrofloxacin and chloramphenicol, for use in PK/PD integration and modelling. A method to grow *Chlamydia* spp. *in vitro* is developed and

used to determine the susceptibility of recently collected, as well as stored, koala isolates of *C. pecorum*. *In vitro* susceptibilities of *C. pecorum* to enrofloxacin, chloramphenicol, and the chloramphenicol derivative, florfenicol, are found to be similar to the susceptibilities reported for other species of *Chlamydia*. The results of this study add further support to the findings presented in Chapters 2 and 3, as the assumed *C. pecorum* susceptibilities used for PK/PD integration and modelling in Chapters 2 and 3 are in close alignment with those determined in this study.

In Chapter 6, the major findings of these studies are highlighted and discussed within the context of what is known about the metabolism of chloramphenicol, enrofloxacin and fluconazole in other mammals. Hypotheses regarding the metabolism of these drugs in koalas are proposed, and recommendations for future studies are discussed.

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GLOSSARY

2-SP	0.2 M sucrose, 0.02 M phosphate
a.k.a	also known as
ATP	adenosine triphosphate
AUC	area under the concentration-time curve
AUC/MIC	area under the concentration-time curve divided by the minimum inhibitory concentration
AUMC	area under the first moment curve
AZWH	Australia Zoo Wildlife Hospital
BGMK	Buffalo Green Monkey Kidney cells
b.i.d.	twice daily
BKWP	Billabong Koala and Wildlife Park
BSV	between subject variability
BW	body weight
CL	clearance
CL/F	apparent clearance
C_{last}	last measured plasma drug concentration
CLSI	Clinical Laboratory Standards Institute
C_{max}	maximum plasma concentration
$C_{\text{max}}/\text{MIC}$	maximum plasma concentration divided by the minimum inhibitory concentration
CNS	central nervous system
CSF	cerebrospinal fluid
C_{ss}	steady state plasma concentration
C_{ssu}	unbound steady state plasma concentration

CTM	Chlamydial transport medium
CYP	cytochrome P450
d.f.	degrees of freedom
DMEM	Dulbecco's Modified Eagle's Medium
EB	elementary body
F	bioavailability
$fAUC_{0-24}/MIC$	unbound area under the curve over 24 h divided by the minimum inhibitory concentration
FBS	foetal bovine serum
fu	unbound drug fraction in plasma
GFR	glomerular filtration rate
HLB	hydrophilic-lipophilic-balanced
HPLC	high performance liquid chromatography
i.m.	intramuscular injection
IQR	interquartile range
IS	internal standard
i.v.	intravenous injection
k_a	absorption rate constant
k_{el}	elimination rate constant
KPS	Koala Hospital of the Koala Preservation Society of Australia
LCAT	latex cryptococcal agglutination test
LLD	lower limit of detection
LLQ	lower limit of quantification
ln	natural logarithm
MBC	minimum bactericidal concentration

MCS	Monte Carlo simulation
MIC	minimum inhibitory concentration
MIC _{TP}	transition point minimum inhibitory concentration
MRT	mean residence time
NaCl	sodium chloride
NSW	New South Wales, Australia
PCR	polymerase chain reaction
PD	pharmacodynamic
PK	pharmacokinetic
PK/PD	pharmacokinetic/pharmacodynamic
p.o.	per os
PSM	plant secondary metabolite
PTA	probability of target attainment
q	every
Qld	Queensland, Australia
qPCR	real time polymerase chain reaction
ρ	Spearman's rho
R ²	coefficient of determination
RB	reticulate body
RSD	relative standard deviation
s.c.	subcutaneous injection
SD	standard deviation
SE	standard error
s.i.d.	once daily
SPE	solid phase extraction

SS	sodium succinate
τ	dosing interval
$T > \text{MIC}$	time for which the drug concentration is above the minimum inhibitory concentration
$t_{1/2}$	half-life
T-PCDE	tannin-protein complex-degrading enterobacteria
t_{last}	time of the last measured plasma drug concentration
t_{max}	time to reach maximum plasma concentration
UKC	uncultured koala <i>Chlamydiales</i>
V_{area}	volume of distribution during pseudoequilibrium
V_{c}	volume of the central compartment
V_{d}	volume of distribution
VG	<i>C. gattii</i> molecular biotype (I-IV)
V_{ss}	volume of distribution at steady state
V_{ss}/F	apparent volume of distribution at steady state

LIST OF PHARMACOKINETIC EQUATIONS

$$t_{1/2} = \ln 2 / k_{el}$$

$$CL = \text{Dose} / AUC_{0-\infty}, \text{ where Dose is administered intravenously.}$$

$$CL/F = \text{Dose} / AUC_{0-\infty}, \text{ where Dose is administered extravascularly.}$$

$$V_{ss} = CL \times MRT$$

$$V_{ss}/F = CL/F \times MRT$$

$$V_{area} = CL / k_{el}$$

$$MRT = AUMC_{0-\infty} / AUC_{0-\infty}$$

$$C_{ss_{average}} = AUC_{0-\infty} / \tau, \text{ where } \tau \text{ is the dosing interval.}$$

$$C_{ssu_{average}} = C_{ss_{average}} \times f_u, \text{ where } f_u \text{ is the unbound fraction in plasma.}$$

$$F = \left(\frac{AUC_{ev}}{AUC_{iv}} \right) \times \left(\frac{Dose_{iv}}{Dose_{ev}} \right)$$

where AUC_{ev} is the AUC determined after extravascular administration, AUC_{iv} is the AUC determined after intravenous administration, $Dose_{iv}$ is the dose administered intravenously, and $Dose_{ev}$ is the dose administered extravascularly.

$$AUC_{extrapolated} = C_{last} / k_{el}, \text{ where } C_{last} \text{ is the last measured plasma concentration.}$$

$$AUMC_{extrapolated} = \left(\frac{C_{last} \times t_{last}}{k_{el}} \right) + \left(\frac{C_{last}}{k_{el}^2} \right)$$

where C_{last} is the last measured plasma concentration, and t_{last} is the time of the last measured plasma concentration.

$$fAUC_{0-\infty} = AUC_{0-\infty} \times \left(1 - \frac{\% \text{ protein binding}}{100} \right)$$

$$\% \text{ protein binding} = \left(\frac{Drug_{total} - Drug_{unbound}}{Drug_{total}} \right) \times 100$$

where $Drug_{total}$ is the concentration of drug added prior to ultrafiltration, and $Drug_{unbound}$ is the concentration of drug in the non-protein bound ultrafiltrate.

Maintenance dose rate = target $C_{ss_{average}}$ x CL, where the target $C_{ss_{average}}$ is extrapolated from minimum inhibitory concentration data.

For allometric scaling:

$y = a \cdot (BW)^b$, where y is the pharmacokinetic parameter being scaled; BW is the median body weight (kg) of koalas used in the analysis; and a and b are the allometric coefficients and exponents.

PUBLICATIONS AND PRESENTATIONS ARISING FROM CANDIDATURE

JOURNAL ARTICLES

Black, L.A., McLachlan, A.J., Griffith, J.E., Higgins, D.P., Gillett, A., Krockenberger, M.B. & Govendir, M. (2012) Pharmacokinetics of chloramphenicol following administration of intravenous and subcutaneous chloramphenicol sodium succinate, and subcutaneous chloramphenicol, to koalas (*Phascolarctos cinereus*). *Journal of Veterinary Pharmacology and Therapeutics*, 36(5):478-485.

Black, L.A., Krockenberger, M.B., Kimble, B., Govendir, M. (2013) Pharmacokinetics of fluconazole following intravenous and oral administration to koalas (*Phascolarctos cinereus*). *Journal of Veterinary Pharmacology and Therapeutics*, 37(1):90-98.

Black, L.A., Landersdorfer, C.B., Bulitta, J.B., Griffith, J.E., Govendir, M. (2013) Evaluation of enrofloxacin use in koalas (*Phascolarctos cinereus*) via population pharmacokinetics and Monte Carlo simulation. *Journal of Veterinary Pharmacology and Therapeutics*, 37(3):301-311.

CONFERENCE ABSTRACTS

Black, L.A., McLachlan, A.J., Govendir, M. (2012) Pharmacokinetics of chloramphenicol in koalas (*Phascolarctos cinereus*) following administration of chloramphenicol sodium succinate. 12th International Congress of the European

Association for Veterinary Pharmacology and Toxicology, Noordwijkerhout, the Netherlands, July 8-12, 2012.

Black, L.A., McLachlan, A.J., Griffith, J.E., Higgins, D.P., Gillett, A., Krockenberger, M.B., Govendir, M. (2012) Pharmacokinetics of chloramphenicol in koalas. Wildlife Disease Association – Australasia Conference, North Stradbroke Island, 23-28 September, 2012.

MANUSCRIPTS COMPLETED FOR SUBMISSION TO JOURNALS

Black, L.A., Higgins, D.P., Govendir, M. *In vitro* activities of chloramphenicol, florfenicol and enrofloxacin against *Chlamydia pecorum* isolated from koalas (*Phascolarctos cinereus*).

CHAPTER 1. INTRODUCTION, LITERATURE REVIEW AND AIMS OF THE THESIS

1.1 INTRODUCTION

The koala (*Phascolarctos cinereus*) is an iconic Australian marsupial that has multiple rehabilitation centres and non-governmental organisations dedicated to its conservation and care (Griffith, 2010). Despite many wild koalas receiving medical treatment in rehabilitation facilities in eastern Australia every year, therapeutic drug dosages for koalas have been extrapolated from physiologically distinct species or formulated by trial and error, and no currently recommended drug dosages have been based on koala-specific pharmacokinetic data. This project was undertaken to determine the pharmacokinetic profiles of three drugs commonly administered to koalas, and to use the pharmacokinetic profiles of each drug in combination with pharmacodynamic principles to assess the likely suitability of currently administered dosages. This general introduction and literature review commences by outlining the distribution and conservation status of the koala, as well as past and present threats to its survival. The aetiological agents, clinical signs, pathological changes and treatment of chlamydiosis and cryptococcosis, the two most prevalent infectious diseases of koalas, are then reviewed. This is followed by a summary of the few pharmacokinetic studies that have been undertaken in the koala to date, and a discussion of the basic principles of pharmacokinetics and pharmacodynamics. Finally, there is a brief discussion of factors that may influence pharmacokinetic profiles in koalas as well as background information on each of the drugs investigated, culminating in the introduction of the specific aims of the study.

1.2 THE KOALA

The koala is an Australian arboreal folivorous marsupial, and the last surviving member of the Family Phascolarctidae. Koalas hold great ecological importance by contributing to Australia's biodiversity and dwelling in a habitat that is home to many other native species (Natural Resource Management Ministerial Council, 2009). With immense public appeal, both locally and internationally, the koala is the most popular Australian wildlife species among international tourists, with one study indicating that 75% of international visitors to Australia hoped to see a koala (Hundloe & Hamilton, 1997). As such, the koala contributes significantly to the Australian economy with a conservative estimate, made in 1997, being in the order of billions of dollars annually, in addition to the provision of thousands of jobs (Hundloe & Hamilton, 1997). Perhaps the clearest demonstration of the extent of public affection for the koala was the South Australian Government's decision to implement an extensive sterilisation and translocation program to address the environmentally damaging problem of koala overpopulation on Kangaroo Island at the turn of the twenty-first century (St John, 1997; Martin & Handasyde, 1999). Culling of other native, over-abundant wildlife species, including the tammar wallaby, the western-grey kangaroo, and the Cape Barren goose, is a commonly employed environmental management strategy on Kangaroo Island (Duka & Masters, 2005; Jackson, 2007), and many other native marsupial species are routinely culled in Australia to manage species overpopulation (Jackson, 2007). However, the national and international public outcry at the prospect of culling koalas on Kangaroo Island was so great that the state government instead opted for a sterilisation and translocation project, involving thousands of koalas and costing A\$1.235 million (Duka & Masters, 2005; Jackson, 2007).

1.3 DISTRIBUTION AND CONSERVATION STATUS

The current distribution of the koala is widespread, yet fragmented (Blanshard & Bodley, 2008), and both the distribution and abundance of koalas have changed since European settlement (Phillips, 1990; Natural Resource Management Ministerial Council, 2009). Prior to European settlement, koalas inhabited the sclerophyll forests of eastern Australia, extending from north-eastern Queensland to Victoria and across to south-eastern South Australia (Natural Resource Management Ministerial Council, 2009). They are also thought to have inhabited six offshore islands at the time of settlement (Jackson, 2007). Initially, koala numbers were reported to have risen dramatically after European settlement; this has been attributed to a concurrent decline in the indigenous population, and a subsequent decrease in hunting pressure (Phillips, 1990). The abundance of koalas during the mid-late nineteenth century was short-lived due to the fur trade, during which they were hunted in immense numbers for their valuable pelts (Lee & Martin, 1988; Martin & Handasyde, 1999). Government legislation to protect the koala from hunting was introduced in Victoria in 1898, and in New South Wales in 1909. The Queensland trade continued intermittently, with the interspersed open and closed hunting seasons. Due to public protest, the final open season was held in Queensland in 1927 (Lee & Martin, 1988). By this time, koalas occupied less than 50% of their previous habitat, and they were considered extinct in South Australia. In Victoria, only a few animals were thought to remain, while the New South Wales koalas were thought to still exist in small remnant populations. In Queensland, the population was noted to have declined (Natural Resource Management Ministerial Council, 2009). Concerns about population crashes induced by the fur trade drove the opening of three koala sanctuaries in Australia in the 1920s and 1930s (Jackson, 2007).

Since the cessation of the fur trade, parts of the koala's former range in Victoria, South Australia, and the Australian Capital Territory have been recolonised with koalas from French Island and Phillip Island. In South Australia, some regions outside of the koala's former range were also repopulated from these island populations (Natural Resource Management Ministerial Council, 2009). In some cases, the introduction of koalas onto islands or to areas outside of their natural range has resulted in overpopulation and overbrowsing, and there are concerns about low genetic diversity in populations that were founded from a few individuals (Houlden et al., 1996; Natural Resource Management Ministerial Council, 2009). Today, koalas still extend from north-eastern Queensland to the South Australian Eyre Peninsula, however, their distribution throughout this range is fragmented. They extend inland past the Great Dividing Range, towards the arid zone of Queensland and New South Wales (Natural Resource Management Ministerial Council, 2009), and into the Australian Capital Territory (Phillips, 1990) (Figure 1-1).

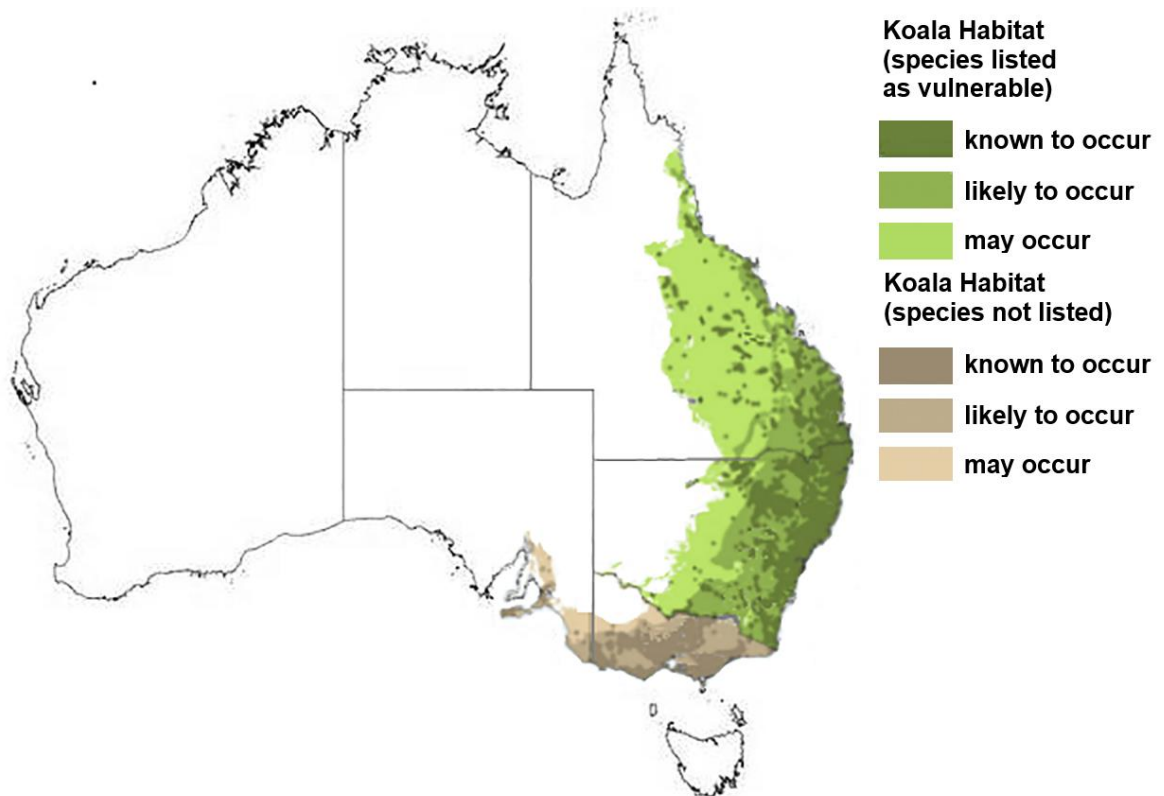


Figure 1-1. Geographical distribution of the koala (Adapted from Department of Sustainability, Environment, Water, Population and Communities, 2013).

The conservation status of the koala varies across its range, with some listings differing between state and federal governments. Under Queensland state legislation, the koala is listed as least concern wildlife, except for the South East Queensland bioregion where it is listed as vulnerable (Natural Resource Management Ministerial Council, 2009; Threatened Species Scientific Committee, 2012). In New South Wales, there are two isolated populations listed as endangered, and koalas throughout the rest of the state are listed as vulnerable (Natural Resource Management Ministerial Council, 2009; Threatened Species Scientific Committee, 2012). In the states of Victoria and South Australia, the koala is not included on the threatened species list (Natural Resource Management Ministerial Council, 2009). In April 2012, the koala was listed as vulnerable throughout Queensland, New South Wales and the Australian Capital Territory under federal environment law. This listing was passed under the

Environmental Protection and Biodiversity Conservation Act 1999, due to a significant population decline throughout this region over three generations (Threatened Species Scientific Committee, 2012; Department of Sustainability, Environment, Water, Population and Communities, 2013).

1.4 THREATS TO THE SPECIES

Despite public awareness and interest in the koala, this iconic species faces a number of threats to its survival. Many of these threats have arisen since European settlement. Destruction, degradation and fragmentation of koala habitat are the result of land-clearing for agriculture and urban development. These habitat threats have been identified as the major threats to the survival of the koala in New South Wales and Queensland, where koala populations are declining (Jackson, 2007, Natural Resource Management Ministerial Council, 2009). South East Queensland currently has one of the fastest growing human populations in Australia (Department of Environment and Resource Management, 2012), and it is little coincidence that koala populations in this region are in decline. Population monitoring in the Koala Coast region of South East Queensland, which is a 375 km² area comprising parts of the Redland, Logan and Brisbane City councils 20 km south-east of Brisbane, has identified a startling 51% decline in koala abundance over the three years from 2005 to 2008 (Department of Environment and Resource Management, 2009), with an overall decline of 68% from 1996 to 2010 (Department of Environment and Resource Management, 2012). Other threats directly related to human activity include motor vehicle strikes and dog attacks. Natural threats to the koala throughout its range include fires, droughts, the effects of climate change, and disease (Gordon et al., 1988; Lee & Martin, 1988; Phillips, 1990; Melzer et al., 2000; Jackson, 2007; Blanshard & Bodley, 2008; Natural Resource

Management Ministerial Council, 2009). Two of the most widely researched and frequently diagnosed infectious diseases to affect koalas are chlamydiosis and cryptococcosis. These diseases underlie the focus of this thesis.

Chlamydiosis is the most common infectious disease of koalas, affecting up to 85% of koalas in some populations (Jackson et al., 1999). A common outcome of chlamydiosis is infertility (McColl et al., 1984; refer to section 1.6.2). Although infertility induced by chlamydiosis is considered unlikely to lead to the extinction of the koala when acting in isolation (Handasyde, 1986; Martin & Handasyde, 1990; Augustine, 1998), modelling suggests that host extinction may occur if chlamydial transmission rates increase in conjunction with pathogenicity, or if non-*Chlamydia* related birth rate decreases or mortality rate increases occur (Augustine, 1998). In support of this model, low fecundity levels in a population in Inverness, Queensland, had a significant effect on population density (Gordon et al., 1990). Additional findings in this population included a negative correlation between chlamydial cystitis and population density, and a reduced survival rate for koalas with cystitis. The authors of this study postulated that in the years of population decline due to reduced fecundity, an additional mortality factor might have had an extreme effect on population size. The historical ability of koala populations to recover from population declines has been attributed to their high reproductive rate (Martin, 1985); however, a decrease in fecundity due to *Chlamydia*-induced infertility has been shown to increase population doubling time in *Chlamydia*-infected koalas compared with their disease-free counterparts (12.2 years and 2.7 – 3.6 years, respectively) (Martin & Handasyde, 1990), which may impact the koala's ability to recover from population declines. Given that the fecundity of *Chlamydia*-infected populations is generally lower than that of disease-free populations, with fertility rates

of 0 – 56% and 59 – 78%, respectively, population declines tend to occur when mortality rates increase (Martin & Handasyde, 1990). Increased habitat destruction and fragmentation can lead to an increased number of animals exposed to edge effects, of which motor vehicle strikes and predation by dogs are likely to be the most important for koalas (Griffith et al., 2013). Thus, edge effects related to habitat loss have the potential to increase mortality rates in affected populations, and coupled with the prolonged population doubling time seen in *Chlamydia*-infected populations, may affect population sizes.

Infectious disease expression in animals results from a complex and dynamic interaction between the host, pathogen and environment. Many koalas carry organisms responsible for disease, including *Chlamydia* spp. and *Cryptococcus* spp., without overt disease expression (Handasyde, 1986; Weigler et al., 1988; Canfield et al., 1991; Connolly et al., 1999; Jackson et al., 1999; Krockenberger et al., 2002b). Stress placed upon individual animals, such as that imposed by loss of habitat, overpopulation, poor nutrition or concurrent disease, can tip the balance in favour of the pathogen and allow clinical disease to ensue. Although cryptococcosis occurs much less frequently than chlamydiosis, clinical disease requires prolonged courses of treatment, and it is a disease that may be seen more frequently as the environmental stressors placed upon koalas, particularly habitat destruction and fragmentation, take their toll.

1.5 THE CHLAMYDIALES

The *Chlamydiaceae* are obligate intracellular bacteria of the Order *Chlamydiales*, and are recognised as important pathogens of humans and animals worldwide. These atypical bacteria have a unique biphasic growth-cycle involving replication inside

eukaryotic cells. The two distinct morphological forms of *Chlamydiaceae* are the infectious elementary body (EB), and the non-infectious reticulate body (RB). The metabolically inactive EB is approximately 0.2 – 0.4 μm (Jones et al., 1997) and is responsible for attaching to and invading target host cells, which are not usually active phagocytes (Moulder, 1991; AbdelRahman & Belland, 2005). Chlamydial EBs are dense units, and possess a rigid cell wall that provides protection from the extracellular environment during host cell lysis and transit between host cells (Beatty et al., 1994). Following host cell infection, the EB internalises itself in a membrane-bound vacuole within the host cell cytoplasm; this is known as the chlamydial inclusion (Moulder, 1991; Hogan et al., 2004; AbdelRahman & Belland, 2005). Within the membrane-bound inclusion, EBs differentiate into metabolically active RBs. These are larger and less dense than EBs, ranging in size from 0.6 to 1.5 μm (Jones et al., 1997). Using energy derived from the host cell in the form of adenosine triphosphate (ATP) (Beatty et al., 1994), RBs multiply by binary fission before reverting to EBs which are released from the host cell by cell lysis or exocytosis (Beatty et al., 1994). During the replicative phase, both EBs and RBs at different stages of replication can be present at any one time (Moulder, 1991). Once liberated from the inclusion, newly formed EBs are able to infect neighbouring cells (Beatty et al., 1994; AbdelRahman & Belland, 2005). In one growth-cycle, a single EB infecting a host cell can give rise to over 1000 new infectious EBs (Moulder, 1991).

Deviations from the typical chlamydial growth-cycle have been observed *in vitro* in association with a number of factors: the presence of certain antibiotics, including penicillin, trimethoprim/sulfamethoxazole and fluoroquinolones (Matsumoto & Manire, 1970; Hammerschlag, 1982; Dreses-Werringloer et al., 2000; Skilton et al., 2009); host

cytokines (Beatty et al., 1993) and other immune system-mediated factors such as monocytes and macrophages (Manor & Sarov, 1988); or changes in the concentrations of nutrients such as amino acids and vitamins (Coles et al., 1993; Raulston, 1997; Harper et al., 2000). This can result in persistent infection, whereby *Chlamydiaceae* can persist within the host cells without apparent growth or replication (Figure 1-2). Whilst in this persistent state, *Chlamydiaceae* are often refractory to antibiotic treatment (Hammerschlag, 2002). Although these aberrant chlamydial forms are viable, they remain culture-negative until the cause of growth suppression is removed (Beatty et al., 1994; Rottenberg et al., 2002). Persistent chlamydial forms hold clinical relevance as the host immune response to recurrent or persistent infections can lead to severe scarring of the infected organs over time (Rottenberg et al., 2002; Stephens, 2003). Chlamydial persistence reflects the difficulties associated with successful elimination of this organism, either by host immune defences or exogenous antimicrobial therapy.

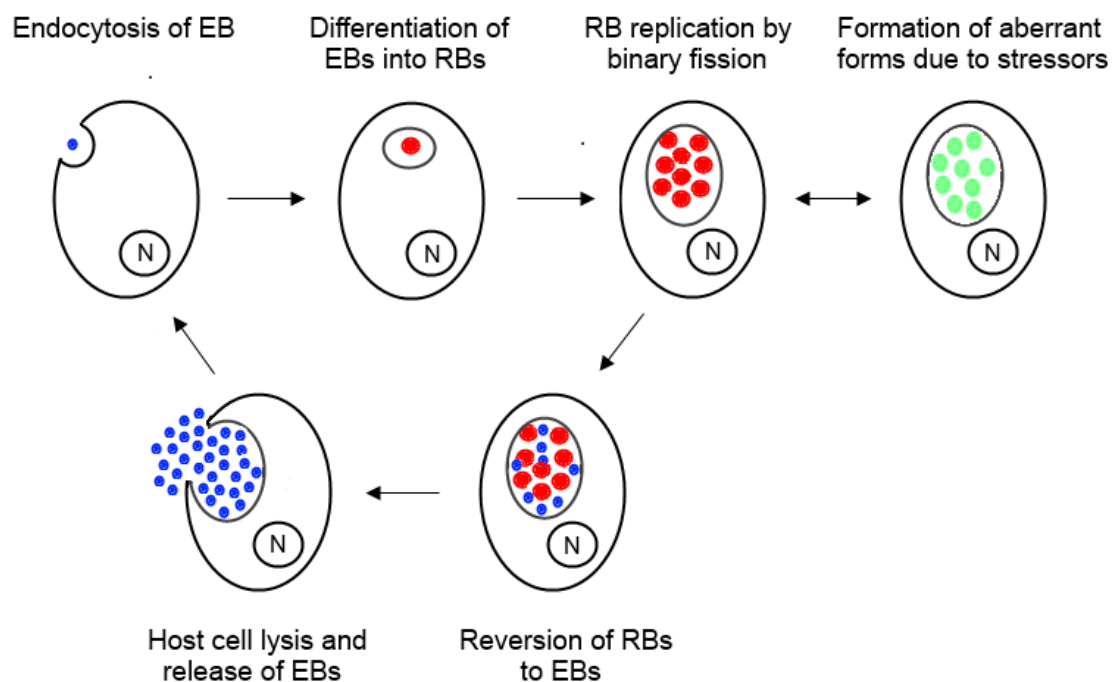


Figure 1-2. The chlamydial life cycle (adapted from Rottenberg et al., 2002). EB: elementary body. RB: reticulate body. N: cell nucleus.

1.6 CHLAMYDIOSIS IN KOALAS

1.6.1 History, taxonomy and epidemiology

Clinical signs consistent with ocular chlamydiosis were first reported as early as 1887 (Troughton, 1973), and reports of urogenital pathology followed (Pratt, 1937). In 1974, koala keratoconjunctivitis was attributed to *Chlamydia* spp. for the first time (Cockram & Jackson, 1974). It was another decade before the chlamydial links to urogenital disease and a rhinitis/pneumonia complex were made (Brown & Grice, 1984; McColl et al., 1984). Chlamydial organisms have been isolated from nasal swabs of koalas with pneumonia and rhinitis using molecular and culture techniques (Brown et al., 1984; Wardrop et al., 1999), yet the results of immunohistochemical staining of the lungs of koalas suffering pneumonitis were inconclusive (Higgins et al., 2005). Thus, the question still remains regarding respiratory tract colonisation versus contamination of nasal surfaces and discharges, either by transient macrophages and monocytes, or from drainage of ocular infections through the nasolacrimal duct (Higgins, 2005).

Initially designated *C. psittaci*, (Cockram & Jackson, 1974; Brown & Grice, 1984; McColl et al., 1984; Girjes et al., 1988), the *Chlamydiaceae* implicated in koala disease have since been reclassified into two species, *C. pecorum* and *C. pneumoniae* (Glassick et al., 1996). A taxonomic revision of the Order *Chlamydiales* in 1999 resulted in a proposal for division into two genera, *Chlamydia* and *Chlamydophila* (Everett et al., 1999), with *C. pecorum* and *C. pneumoniae* being included in the new genus, *Chlamydophila*. However, this proposal was not widely adopted. The commonly accepted taxonomy remains a single genus, *Chlamydia* (Kuo & Stephens, 2011), containing nine species (Kuo et al., 2011); this is the taxonomy that will be used throughout this thesis. In addition to *C. pecorum* and *C. pneumoniae*, genotypes of nine

novel *Chlamydia*-like bacteria have been identified in urogenital and ocular swabs taken from koalas. These have been designated the uncultured koala *Chlamydiales* (UKC), and tend to occur as co-infections with *C. pecorum* and *C. pneumoniae*; although their role in disease is unclear, there is speculation they may be implicated in ascending urinary tract infections (Devereaux et al., 2003).

Chlamydiosis has been detected in koala populations throughout their range (McKenzie, 1981; Obendorf, 1983; Weigler et al., 1988; Jackson et al., 1999; Wardrop et al., 1999; Griffith & Higgins, 2012), although not all infected koalas show clinical disease. *C. pecorum* appears to be more pathogenic than *C. pneumoniae*, causing infections of a higher severity and at a higher frequency than the latter (Jackson et al., 1999). Sole *C. pneumoniae* infections are frequently low-grade or asymptomatic (Jackson et al., 1999; Devereaux et al., 2003; Griffith, 2010). The mode of transmission has not been formally studied, although there is strong evidence for venereal transmission (Obendorf, 1983; Handasyde, 1986; Girjes et al., 1988). Faecal-oral transmission to dependent young during pap feeding and direct transfer of infected ocular and urogenital discharges between koalas have also been suggested (Blanshard & Bodley, 2008).

1.6.2 Clinical signs and pathological changes associated with chlamydiosis

The range of body systems affected by chlamydiosis accounts for the variation in clinical signs and pathological changes associated with disease. Ocular infection may result in unilateral or bilateral disease, although chronic cases are usually bilateral. Clinical signs include excessive lacrimation, blepharospasm, a serous to mucopurulent ocular discharge, hyperaemia and proliferation of the conjunctiva and nictitating membrane, chemosis, keratitis, pannus formation, corneal oedema, and forced closure

of the eyelids due to excessive discharge (Cockram & Jackson, 1981; Brown & Grice, 1986; Kempster et al., 1996). Secondary bacterial infections and trauma to the eyelids due to excessive rubbing may be seen (Obendorf, 1983). Koalas with urogenital chlamydiosis often show clinical signs of cystitis, including gross haematuria, pollakiuria, and dysuria; the latter may be identified by grunting or cloacal eversion during micturition. Urinary incontinence may be identified as a lack of posturing for micturition and by the greasy, brownish pelage staining of the rump, for which the condition is colloquially termed ‘wet bottom’ or ‘dirty tail’ (Figure 1-3). The skin may be inflamed and ulcerated as a result of urine scalding, and cutaneous myiasis may be present (Obendorf, 1983; Brown et al., 1987; Blanshard & Bodley, 2008). Affected koalas may be dehydrated, with muscle wasting and signs of depression (Canfield, 1990). The sparse data regarding chlamydial respiratory disease in koalas describe a serous to purulent nasal discharge, sneezing, coughing, and dyspnoea as the clinical signs (Brown & Grice, 1986; Wardrop et al., 1999).



Figure 1-3. Rump pelage staining due to urinary incontinence associated with urogenital tract chlamydiosis (Author's own image).

Histopathologically, transmigration of polymorphonuclear cells may be seen across capillary walls of the affected organs in the acute stage of chlamydiosis, with more chronic disease being characterised by a lymphocytic-plasmacytic infiltration (Kempster et al., 1996), sometimes with monocytes and neutrophils (McColl et al., 1984; Obendorf & Handasyde, 1990; Hemsley & Canfield, 1997). The normal architecture of the affected tissue is eventually replaced with fibrous connective tissue (Obendorf & Handasyde, 1990), rendering affected organs dysfunctional and causing profound morbidity in some animals. Although affected females with reproductive tract disease may not show overt signs of disease, they are often infertile (Blanshard & Bodley, 2008). The ovaries tend to retain cyclical activity, containing follicles at various stages of development, corpora lutea, and degenerate remnants of corpora lutea (Obendorf, 1981). This suggests that infertility associated with chlamydiosis is due to

fibrosis of the reproductive tract rather than a lack of cyclic ovarian activity. In order to prevent the chronic changes associated with chlamydiosis, effective antimicrobial treatment is required early in the course of disease prior to the development of the irreversible fibrosis that occurs with chronic disease.

1.6.3 Treatment of chlamydiosis

1.6.3.1 Topical therapy

Topical therapy is usually administered when affected koalas are presented to wildlife rehabilitation facilities with conjunctivitis. Topical therapy often consists of commercial eye ointments containing antimicrobials with anti-chlamydial activity, including oxytetracycline, chloramphenicol and ofloxacin. A topical corticosteroid may also be applied, and the duration of therapy varies between rehabilitation facilities. Topical therapy may be accompanied by conjunctival ablation in eyes with severe conjunctival proliferation, or in cases that are refractory to treatment (Blanshard & Bodley, 2008).

Topical therapy for chlamydial conjunctivitis is often accompanied by systemic antimicrobial therapy, typically enrofloxacin or chloramphenicol (Blanshard & Bodley, 2008). In some cases of chlamydial keratoconjunctivitis, systemic antimicrobial therapy is not administered despite evidence that keratoconjunctivitis is often accompanied by clinical or subclinical urogenital disease (Griffith, 2010), and that topical treatment does not prevent chlamydial shedding from other infected sites (Markey et al., 2007).

1.6.3.2 Systemic therapy

Systemic therapy is administered for the treatment of urogenital disease, and in some facilities it is used in conjunction with topical treatment for keratoconjunctivitis. The

systemic treatment of chlamydiosis is complicated by the koala's intolerance of the first line anti-chlamydial drugs used in humans and other species, the macrolides (e.g. erythromycin, azithromycin) and tetracyclines. Oxytetracycline and erythromycin have been found to induce a syndrome of inappetence, wasting, and death following systemic administration to koalas (Brown et al., 1984; Osawa & Carrick, 1990). More recently, a clinical trial testing newer forms of the macrolides and tetracyclines, azithromycin and doxycycline, was undertaken in koalas suffering chlamydiosis. All koalas receiving azithromycin orally (p.o.; n = 3) and five of six koalas receiving doxycycline [either intramuscularly (i.m.) once weekly, or p.o. once daily (s.i.d.) or twice daily (b.i.d.)] displayed signs of depression, inappetence, weight loss, and decreased faecal output. There was no response to various rescue treatments (including gastrointestinal prokinetic agents, vitamin B injections, oral administration of fresh caecal contents or pulverised *Eucalyptus* spp. leaf, soya-based supplements, or parenteral fluid therapy) and all koalas were euthanased on welfare grounds, except for one that died prior to euthanasia (Griffith, 2010).

The koala's intolerance of these antimicrobials may be due to disruption of the tall, dense fringe of bacteria that is closely adhered to the caecal mucosal surface. This bacterial layer is arranged in long parallel chains, with an orientation perpendicular to the caecal wall (McKenzie, 1978). The bacterial fringe consists of numerous morphologically distinct bacteria, including gram positive cocci and bacilli, gram negative bacilli, both gram positive and gram negative fine branching filaments thought to be actinomycetes, and tannin-protein complex-degrading enterobacteria (T-PCDE) (McKenzie, 1978; Osawa, 1992; Osawa et al., 1993). The latter are involved in degradation of tannin-protein complexes that are abundant in the koala's *Eucalyptus*

spp. diet, thus increasing digestibility of their diet by liberating nitrogen in the form of ammonia, which can be used by microflora for protein synthesis (Osawa et al., 1993). One study demonstrated the absence of the caecal microbial lining following a striking loss of over 30% body weight and subsequent death in a koala receiving systemic oxytetracycline for cystitis (Osawa et al., 1993), implicating the loss of these important gastrointestinal microflora in the pathogenesis of the wasting syndrome seen with systemic oxytetracycline use. Loss of T-PCDE from the caecal wall may render affected koalas unable to assimilate their highly indigestible *Eucalyptus* spp. diet, resulting in malnutrition and death (Osawa & Carrick, 1990).

Supplementary feeding with a soya-based product during and following systemic oxytetracycline therapy for chlamydiosis has shown promising results. Despite some weight loss occurring during treatment, weight was regained following treatment and a successful return to a eucalypt-only diet was reported (Osawa & Carrick, 1990). Weekly doxycycline injections have also shown promise for keratoconjunctivitis when accompanied by supplementary feeding (Blanshard, 1994). However, this approach has not gained wide acceptance and alternative antimicrobial therapies have been adopted instead.

Current suggested systemic antimicrobial therapy for chlamydiosis typically consists of enrofloxacin 10 mg/kg s.i.d. subcutaneously (s.c.), or chloramphenicol 60 mg/kg s.i.d. s.c. (Blanshard & Bodley, 2008). Until recently, enrofloxacin was also administered p.o. (Blanshard & Bodley, 2008). However, very low plasma concentrations of enrofloxacin have been reported following oral administration to koalas (Griffith et al., 2010), thus s.c. treatment is now more widely adopted. There are anecdotal reports of success with

chloramphenicol 30 mg/kg b.i.d. s.c. (Blanshard, 1994; Blanshard & Bodley, 2008), although this protocol is not used widely.

Recent studies have shed some light on the suitability of these therapies for treating koalas with chlamydiosis. Four to ten weeks of treatment with enrofloxacin at 10 mg/kg s.c. s.i.d. or 20 mg/kg p.o. s.i.d., or with marbofloxacin at 10 mg/kg p.o. s.i.d., led to resolution of clinical signs and cessation of mucosal chlamydial shedding by the end of the treatment course, as detected by real-time polymerase chain reaction (qPCR) (Griffith, 2010). However, rebound shedding of *C. pecorum*, or *C. pecorum* and *C. pneumoniae*, occurred following treatment withdrawal in 24 of 29 (83%) koalas. This indicates treatment failure in the majority of cases, and raises questions regarding the suitability of the fluoroquinolones, enrofloxacin and marbofloxacin, in treating koalas with chlamydiosis at these dose rates. By contrast, chloramphenicol treatment trials indicated a dosage of 60 mg/kg s.c. s.i.d. stopped chlamydial shedding by the end of the treatment course (which lasted up to 45 days, but often shedding ceased earlier) as well as a continued absence of chlamydial shedding for two (Markey et al., 2007) or seven (Govendir et al., 2012) weeks following treatment withdrawal, as detected by qPCR.

1.7 THE *CRYPTOCOCCUS NEOFORMANS* SPECIES COMPLEX

The *Cryptococcus neoformans* species complex consists of *C. neoformans* var. *neoformans*, *C. neoformans* var. *grubii*, and *C. gattii*. These basidiomycetous fungi cause disease in many animal species worldwide, including humans, and clinical presentation can vary dramatically between host species (Lin & Heitman, 2006). In clinically affected humans and animals, *Cryptococcus* spp. exists as a yeast; this is the

unicellular asexual form, which reproduces by budding. Although *Cryptococcus* spp. can reproduce sexually, this state is not documented to be pathogenic (Casadevall & Perfect, 1998).

1.8 CRYPTOCOCCOSIS IN KOALAS

1.8.1 History and Taxonomy

Cryptococcosis was first detected in koalas in 1959 (Backhouse & Bolliger, 1960). The three cases reported at this time were among the first non-human cases of cryptococcosis to be reported in Australia. Sporadic cases were subsequently reported, until a comprehensive review of 43 cases was published in 2003 (Krockenberger et al., 2003). This helped to shed light on the previously poorly described spectrum of clinical disease seen in koalas with cryptococcosis. Prior to this review, only 11 cases had been published, with the predominant clinical presentations being consistent with respiratory and neurological disease. Two of these cases originated from captive koalas in Western Australia (Gardiner & Nairn, 1964), and the remainder were from wild and captive koalas within their natural range along the eastern Australian seaboard, predominantly within and surrounding Sydney (Backhouse & Bolliger, 1960; Backhouse & Bolliger, 1961; Bolliger & Finckh, 1962; Canfield et al., 1986; Spencer et al., 1993; Malik et al., 1997; Woods & Blyde, 1997).

Since the first human case of cryptococcosis was reported in 1894 (Viviani & Tortorano, 2009), the *Cryptococcus neoformans* species complex has undergone a number of taxonomic reclassifications. By the 1980s, a single species containing two varieties and five serotypes had gained acceptance. These were *C. neoformans* var. *neoformans*, which comprised serotypes A, D, and the hybrid AD, and *C. neoformans*

var *gattii*, containing serotypes B and C (Kwon-Chung et al., 1982; Casadevall & Perfect, 1998). In 1999, serotypes A and D were separated into different varieties: *C. neoformans* var *neoformans* (serotype D) and *C. neoformans* var *grubii* (serotype A) (Franzot et al., 1999). A more recent separation based on molecular differences saw serotypes B and C reclassified as a distinct species, *C. bacillisporus* (Boekhout et al., 2001), which was subsequently renamed *C. gattii* (Kwon-Chung et al., 2002). Thus, the currently used nomenclature, and that used throughout this thesis, is *C. neoformans* (var. *neoformans* and var. *grubii*) and *C. gattii*. There are four molecular biotypes of *C. gattii*, designated VGI to VGIV (Ellis et al., 2000).

1.8.2 Epidemiology

Although a relatively uncommon disease, cryptococcosis is possibly more common in koalas than in any other species (Krockenberger et al., 2003; Malik et al., 2011), with clinical disease identified in 2.5% of cases in a post mortem examination series carried out at The University of Sydney between 1980 and 2003 (Stalder, 2003). Members of the *Cryptococcus neoformans* species complex are ubiquitous in the environment, with *C. neoformans* commonly isolated from pigeon guano (Emmons, 1955) and soil (Emmons, 1951), and the VGI biotype of *C. gattii* being closely associated with *Eucalyptus* spp. trees, particularly *E. camaldulensis* and *E. tereticornis* (Ellis & Pfeiffer, 1990; Pfeiffer & Ellis, 1992; Chakrabarti et al., 1997; Krockenberger et al., 2002b). These two species are the preferred food trees by koalas in some regions (Gordon et al., 1988; Gordon et al., 1990; White & Kunst, 1990). *C. gattii* has been identified as the causative agent in all of the koala cryptococcosis cases (29 out of 43) that have been biotyped, and at least nine of the remaining cases are likely to have been caused by *C. gattii*, based on histopathological or morphological evidence. For the five remaining

cases, there was insufficient information for an assessment to be made (Krockenberger et al., 2003). VGI is responsible for the vast majority of koala cryptococcosis cases (Malik et al., 2011). A small number of clinical cases caused by VGII have been detected in captive koalas in Western Australia (Malik et al., 2011), where this biotype tends to be more prevalent and may be more virulent than VGI (McGill et al., 2009).

The contact between koalas and *Cryptococcus* spp. has three known outcomes: nasal colonisation only, whereby *Cryptococcus* spp. may be isolated in the absence of a positive serum cryptococcal antigen titre (Connolly et al., 1999); subclinical disease, detected as a positive cryptococcal antigen titre in the absence of clinical disease (Krockenberger et al., 2002a); and clinical disease, whereby affected animals show clinical signs of disease and have relatively high cryptococcal antigen titres (Krockenberger et al., 2002a). Although clinical cryptococcosis appears to be a relatively uncommon outcome of the complex interaction between the host, pathogen and environment, colonisation of the skin and upper respiratory tract by *C. gattii* occurs commonly in koalas, with nasal colonisation being detected in up to 100% of koalas in some captive groups (Krockenberger et al., 2002b). Colonisation by *C. neoformans* also occurs, but not as often (Connolly et al., 1999; Krockenberger et al., 2002b). The close affiliation between *C. gattii* VGI and *Eucalyptus* spp. offers a plausible explanation for the increased rates of disease and colonisation caused by this species, compared with *C. neoformans* (Connolly et al., 1999; Krockenberger et al., 2002b). This is supported by the finding of increased nasal colonisation and subclinical disease prevalence in environments that carry high burdens of *C. gattii* (Krockenberger et al., 2002a; Krockenberger et al., 2002b). Additionally, there is evidence that koalas can amplify numbers of *C. gattii* in their environment (Krockenberger et al., 2002b), which is an

intriguing finding that may be related to the prevalence of cryptococcosis in this species.

Subclinical disease has the potential to spontaneously resolve, remain subclinical, or progress to clinical disease, and all three outcomes have been identified in koalas (Krockenberger et al., 2002a). There is no indication that cryptococcosis is zoonotic or contagious (Sorrell, 2001), and disease caused by *C. gattii* usually manifests in hosts without overt immunocompromise; by contrast, *C. neoformans* usually infects immunocompromised hosts (Rozenbaum & Gonçalves, 1994; Chen et al., 2000). There is strong evidence supporting a respiratory route of infection in humans (Casadevall & Perfect, 1998), although primary cutaneous inoculation has also been reported (Hamann et al., 1997); both of these routes of infection are suspected in koalas.

1.8.3 Clinical signs and pathological changes associated with cryptococcosis

Clinically, koalas with cryptococcosis tend to present with signs of respiratory tract disease (upper or lower) or neurological signs. Nasal discharge is commonly associated with disease of the rostral nasal cavity, whereas stertorous breathing, dyspnoea and sometimes aerophagia are associated with disease of the caudal nasal cavity and nasopharynx. Facial distortion may occasionally be present in cases of rostral nasal cavity disease. Pulmonary disease tends to manifest as severe dyspnoea. Some affected animals will present deceased with no identifiable cause of death, and affected koalas are frequently in poor body condition due to the chronic course of disease (Krockenberger et al., 2003).

Seizures are a common clinical presentation for koalas with neurological disease (Krockenberger et al., 2003), and blindness, nystagmus, rolling, circling, and paresis progressing to paralysis have been described (Backhouse & Bolliger, 1960; Backhouse & Bolliger, 1961; Bolliger & Finckh, 1962; Spencer et al., 1993).

Histopathology of affected tissues reflects a spectrum of host responses. Some cases display minimal host response, others show evidence of acute inflammation characterised by neutrophils, and others have more chronic pathological changes reflective of pyogranulomatous and granulomatous inflammation (Krockenberger et al., 2003).

1.8.4 Treatment of cryptococcosis

There are only a few reports regarding treatment of cryptococcosis in koalas. Early recommendations included ketoconazole 10 mg/kg s.i.d. p.o., itraconazole 20 - 40 mg/kg s.i.d. or b.i.d. p.o., fluconazole up to 100 mg b.i.d. p.o., or fluconazole 3 mg/kg s.i.d. p.o. following a loading dose of 6 mg/kg on the first day of treatment (Blanshard, 1994; Booth & Blanshard, 1999).

A number of clinical reports of ketoconazole therapy have been made (Blanshard, 1994). Interpretation of these reports is confounded by the following factors: a koala with a positive cryptococcal antigen titre (LCAT) that failed to respond to therapy had pneumonia and was receiving concurrent chloramphenicol and gentamicin, so may have succumbed regardless of whether ketoconazole treatment was successful; a koala receiving ketoconazole for cutaneous nodules with a positive culture of *C. gattii* and a positive LCAT yielded a negative LCAT following ketoconazole therapy (10 mg/kg

p.o. s.i.d.), but resolution of the skin disease was attributed to topical silver nitrate; and a clinically normal koala with a positive LCAT (1:120) yielded a negative LCAT following ten days of ketoconazole treatment at 10 mg/kg p.o. s.i.d. (Blanshard, 1994). Although this small set of cases indicates ketoconazole may be effective in reducing a positive LCAT titre, more information is required to enable an assessment of clinical response to therapy. Additionally, there is anecdotal evidence that positive LCATs can resolve spontaneously over time (Krockenberger et al., 2002a), making response to treatment of subclinical disease difficult to assess.

Itraconazole therapy has reportedly cured cryptococcal lesions in koalas following 90 days of therapy, although the dosage and location of the lesions were not reported (Booth & Blanshard, 1999). More recently, combination therapy has been recommended for cases of cryptococcosis (Blanshard & Bodley, 2008; Malik et al., 2011), and is based on regimens that were developed for cats (Malik et al., 2001) in an attempt to overcome amphotericin B associated nephrotoxicity. The protocol involves twice-weekly administration of subcutaneous amphotericin B (0.5 mg/kg diluted in 300 mL 2.5% dextrose and 0.45% NaCl), in conjunction with an orally administered triazole (itraconazole 100 mg/day or fluconazole 50-100 mg b.i.d.), and has been used with some success (Krockenberger et al., 2003; Blanshard & Bodley, 2008). This protocol has been used in koalas without nephrotoxic side effects being observed, although monitoring renal function throughout therapy is recommended (Blanshard & Bodley, 2008). Treatment duration may range from 2 to 18 months, depending on disease severity. Antifungal concentrations in plasma should be monitored and appropriate dosage adjustments made in cases that are responding poorly to therapy. Monthly

LCATs should be monitored, and treatment should be continued until a negative LCAT persists for at least one month (Blanshard & Bodley, 2008).

A recent case study reported a lack of recurrence of a cryptococcal granuloma in the nasal sinus, as determined by computed tomography, during combination therapy with itraconazole 10 mg/kg s.i.d. p.o. (reduced from 10 mg/kg b.i.d. p.o. based on therapeutic monitoring of plasma concentrations) and amphotericin B (initially s.c. as per the above protocol, then intralesionally following debulking surgery) (Wynne et al., 2012). Although this koala was showing promising results with combination therapy, the koala maintained an elevated LCAT so no indication was provided as to whether the disease resolved with this therapy.

In another case whereby a koala received fluconazole 100 mg p.o. b.i.d. and amphotericin B (0.5 mg/kg in 60 mL Hartmann's solution) three times weekly, the LCAT continued to rise despite nasal swabs becoming negative for *C. gattii* during therapy. This koala was changed from fluconazole to ketoconazole 50 mg p.o. b.i.d., and the amphotericin B was continued. At the time of reporting, there was evidence of clinical improvement following three months of therapy, although the LCAT was not yet negative (Woods & Blyde, 1997). This koala subsequently died in July 1998 (Krockenberger et al., 2003).

1.9 THERAPEUTIC DRUG STUDIES IN KOALAS

Prior to 2010, there were no pharmacokinetic data available for any therapeutic drug used to treat koalas. For this reason, therapeutic drug dosage regimens have been developed by extrapolation from other species, including humans (Blanshard & Bodley,

2008). Many of these extrapolated dosages have gained acceptance as they appear to be well-tolerated by koalas in most cases (Blanshard & Bodley, 2008). However, this does not address the question of whether the dosage is sufficient for treatment of the relevant condition. Although the consideration of drug pharmacokinetics in koalas is relatively new, the few studies published to date have revealed some interesting and important findings that hold implications for therapeutic drug use in this species.

The first koala pharmacokinetic study involved subcutaneous and oral administration of the fluoroquinolones, enrofloxacin and marbofloxacin, to koalas with chlamydiosis (Griffith et al., 2010). This study demonstrated low bioavailability of oral enrofloxacin relative to subcutaneous administration, and absorption rate-limited disposition of the oral formulation when compared to the subcutaneous formulation. Bioavailability and absorption rate could not be further qualified due to the lack of an intravenous (i.v.) treatment group. A subsequent study investigated chloramphenicol pharmacokinetics and its effect on chlamydial shedding when administered subcutaneously to koalas with chlamydiosis (Govendir et al., 2012). This study concluded that although chloramphenicol prevented chlamydial shedding for up to 63 days post treatment-withdrawal in the majority of koalas, the plasma concentrations attained at 60 mg/kg s.i.d. s.c. were below target therapeutic concentrations. These studies had two common limitations. Firstly, neither study included an i.v. treatment group, which precluded estimation of important pharmacokinetic parameters, namely absolute bioavailability (F), volume of distribution (V_d), and systemic clearance (CL). Secondly, the therapeutic targets in both cases were deduced from data derived using other antimicrobial-pathogen combinations, as no data existed regarding the *in vitro* susceptibility of koala isolates of *C. pneumoniae* or *C. pecorum* to any antimicrobial agent.

The most extensive pharmacokinetic study published to date investigated the pharmacokinetic profile of the non-steroidal anti-inflammatory drug, meloxicam (Kimble et al., 2013). Not only was meloxicam poorly orally absorbed by koalas in the study, it was also rapidly eliminated. This study highlighted the clinical implications of the koala's ability to metabolise and eliminate xenobiotics, and emphasised the need to perform pharmacokinetic studies in koalas to enable evaluation of the likely efficacy of commonly used drugs. Importantly, this study demonstrated the inherent flaws associated with dose extrapolation between physiologically distinct species, namely the small carnivores (dogs and cats) from which many koala drug doses have been extrapolated, and koalas.

These studies have demonstrated the importance of pharmacokinetic studies in species that have unique metabolic needs, such as the koala. Hundreds of koalas are presented to individual wildlife hospitals on the east coast of Australia for medical treatment every year (Jones, 2008; Griffith et al., 2013), which would equate to thousands being treated annually across their natural range. This makes pharmacokinetic studies crucial in determining which drugs are likely to have therapeutic benefit in koalas, and which are not. Not only are the time and money that go into treating sick koalas important, but the animal welfare implications of hospitalising animals to administer drugs without data on their likely efficacy must be considered, as well as the dangers of antimicrobial resistance development due to the use of subtherapeutic antimicrobial dosages.

1.10 PHARMACOKINETICS AND PHARMACODYNAMICS: A

SUMMARY OF BASIC PRINCIPLES

The integration of drug pharmacokinetics and pharmacodynamics is important in therapeutic drug development and dosage design. Pharmacokinetics pertains to the study of the time-course of drug molecules in the body, as described by mathematical models. It describes how an animal processes a drug following administration, encompassing four key physiological processes: absorption, distribution, metabolism, and elimination. Together, these constitute drug disposition (Riviere, 2009a; Riviere, 2009b). Pharmacodynamics characterises the relationship between the drug concentration at target receptors within the body and the resulting physiological effect, if one exists. Pharmacokinetic/pharmacodynamic (PK/PD) integration thus describes the correlation between the drug concentration in the bloodstream, the drug concentration at the therapeutic target site, and the clinical outcome (Martinez et al., 2006a). For antimicrobial drugs, this enables specific antimicrobial-pathogen combinations to be evaluated and provides a guide for estimating dosages to optimise clinical and microbial outcomes (Martinez et al., 2006a).

1.10.1 Protein binding

PK/PD studies tend to be based on concentrations of free drug (i.e., the portion not bound to plasma proteins) in the serum or plasma. Measurement of plasma protein binding is an important component of pharmacokinetic studies as it enables the free or unbound drug concentration in the bloodstream to be determined. This is the drug fraction that is available to exhibit its pharmacologic effects, undergo metabolism, and be excreted from the body. The reasons for sampling drug concentrations in serum or plasma are numerous. Firstly, following administration, drugs enter the bloodstream in

which they are transported throughout the body. Secondly, peripheral blood samples are minimally invasive to obtain. Finally, many drugs reach concentrations in the systemic circulation that are equivalent to extracellular concentrations in well-perfused tissues, meaning serum or plasma concentrations are often indicative of tissue concentrations (Riviere, 2009a).

1.10.2 Absorption, first-pass metabolism, and bioavailability

Therapeutic drugs can be administered systemically by a number of routes; these include, but are by no means limited to, oral administration and parenteral administration. Parenteral routes include subcutaneous, intramuscular, intravenous, inhalational and transcutaneous administration. For drugs that are administered to elicit systemic effects, the drug must move from the site of administration into the systemic circulation following extravascular administration. This is the process of absorption (Riviere, 2009a). Following oral administration, drug absorption may be affected by the dosage form (eg oral solution, suspension, capsule, tablet, or paste); the presence of ingesta in the proximal gastrointestinal tract, which may influence gastric-emptying time or provide opportunities for drug-chelation to divalent cations or other feed constituents; pre-systemic or first-pass metabolism by microorganisms within the gastrointestinal tract, or drug metabolism by enzymes within the enterocytes themselves; the presence of efflux transporter proteins, such as P-glycoprotein, that expel drugs back into the intestinal lumen following absorption; and the physicochemical properties of the drug (Baggot, 2006; Riviere, 2009a). Once in solution, drugs must be relatively lipid-soluble and in a non-ionised form for successful passive absorption across the phospholipid bilayer cell membranes of the gastrointestinal mucosa; alternatively, some drugs are absorbed by active transport

mechanisms (Schanker, 1962; Kararli, 1989; Kwon, 2001). Drug absorption that occurs distal to the oral cavity and proximal to the rectum results in the drug being present in the portal circulation. This is true for the majority of drugs, as the primary site of gastrointestinal drug absorption is the small intestine. Prior to entry into the systemic circulation, absorbed drug in the portal circulation must pass through the liver where first-pass metabolism may occur (Baggot, 2006; Riviere, 2009a).

Drugs administered via intramuscular or subcutaneous routes are not subject to first-pass metabolism, as the capillaries that transport these drugs from the tissues drain directly into the systemic circulation. Intramuscular and subcutaneous administration routes also evade many of the other barriers to absorption faced by orally administered drugs. The main factors influencing absorption from intramuscular or subcutaneous injection sites are the degree of vascularity at the injection site, the formulation of the injectable preparation, and the physicochemical properties of the drug (Baggot, 2006; Riviere, 2009a).

The extent of drug absorption following extravascular administration is reflected in a drug's systemic bioavailability (F), which is the fraction of an administered dose that reaches the systemic circulation unchanged (Riviere, 2009a). Bioavailability can range from 0.0, whereby none of the drug reaches the systemic circulation, to 1.0, whereby 100% of the administered drug reaches the systemic circulation. Drugs administered i.v. have a bioavailability of 1.0 as the entire dose is deposited directly into the systemic circulation, avoiding an absorption phase in the drug disposition profile. Thus, plasma disposition of an i.v. dose needs to be known in order to assess the bioavailability of an extravascular dose (Birkett, 2002; Riviere, 2009a). Incomplete bioavailability following

oral administration is a common finding, and can be attributed to the previously mentioned factors that affect oral drug absorption. Following intramuscular or subcutaneous administration, incomplete bioavailability may be due to some degree of precipitation of the administered formulation at the injection site, or to tissue irritation caused by a component or property of the administered formulation (Baggot, 2006). As drug disposition following i.v. administration is not confounded by absorption, the i.v. route is used to determine the pharmacokinetic parameters of a drug (Baggot, 2006), and comparisons can then be made to other routes of administration.

1.10.3 Drug distribution

Following absorption into the systemic circulation, the free drug fraction may be distributed to the extravascular tissues, including the organs responsible for drug metabolism and elimination. Drug distribution is governed by a number of factors, including the degree of binding to plasma proteins; the physicochemical properties of the drug, which determine its ability to penetrate cellular barriers and its affinity for tissues; the degree of tissue perfusion; and the concentration gradient between blood and tissue. Many drugs are distributed to tissues through capillary pores, or by diffusion down a concentration gradient. Tissue binding may occur, and this may increase the distribution of a drug. This does not always correlate with an increase in drug activity, as the site of sequestration may not be the site of drug activity (Baggot, 2006; Riviere, 2009a).

1.10.4 Metabolism and elimination

The processes of metabolism and elimination are closely related, and may vary between drugs and animal species. The two organs responsible for the vast majority of

metabolism and elimination processes are the liver and the kidney. Some agents are almost entirely eliminated via renal excretion, whilst others are eliminated by hepatic metabolism followed by biliary or renal excretion (Baggot, 2006). Lipophilic drugs generally undergo hepatic metabolism into more hydrophilic metabolites prior to excretion. Hepatic metabolism is broadly classified into two phases, termed Phase I and Phase II metabolism. There are four types of reactions involved in Phase I metabolism: oxidation, reduction, hydrolysis, and hydration. These reactions add a functional group to the parent molecule. The products of Phase I metabolism are subsequently subject to Phase II conjugation reactions to enable deactivation of these metabolites. Phase II metabolism usually involves conjugation of the functional group that was added during Phase I metabolism, although Phase II metabolism also plays an important role in the metabolism of some parent drugs that have no requirement for Phase I metabolism (Riviere, 2009a). The most widely studied of the many hepatic metabolic pathways are the cytochrome P450-mediated (CYP) reactions. These vary widely between species and some of these have been studied in koalas (refer to Section 1.11).

Of those drug metabolites and parent drugs that are secreted into the bile, some are excreted in the faeces. Others may undergo a process known as enterohepatic recirculation, whereby the drug or its metabolite may be reabsorbed by the small intestine, transported back to the liver and either resecreted into the bile, or excreted by the kidney after reaching the systemic circulation. In some cases, enterohepatic recirculation involves hepatic conjugation and intestinal bacterial deconjugation of a drug, prior to its reabsorption in the small intestine (Roberts et al., 2002; Riviere, 2009a). Enterohepatic recirculation may affect the pharmacokinetic profile of a drug, usually by prolonging drug elimination (Roberts et al., 2002).

1.10.5 Systemic clearance

The disposition of a drug can be described mathematically by its pharmacokinetic parameters. The two parameters that are fundamental to the description of drug disposition are the systemic CL and the V_d (Baggot, 2006). The systemic CL signifies the volume of blood or plasma from which a drug is cleared per unit time (Baggot, 2006), indicating the ability of the body to irreversibly eliminate a drug following administration. Systemic CL is the most important pharmacokinetic parameter as it controls overall drug exposure, and is necessary for determination of the dosage regimen required for maintenance of a selected steady state plasma concentration (Toutain & Bousquet-Mélou, 2004a).

1.10.6 Volume of distribution

V_d is a proportionality constant that relates the plasma drug concentration to the total amount of drug in the body (Toutain & Bousquet-Mélou, 2004b; Baggot, 2006), and can be represented by the following equation:

$$V_d = \frac{\text{total amount of drug in the body at time } t}{\text{drug plasma concentration at time } t}$$

This ratio varies depending on the state of drug disposition. To allow for this, there are three V_d terms that can be estimated: the volume of the central compartment (V_c), which is calculated as the initial volume of distribution following i.v. bolus administration, and represents the apparent volume from which drug elimination occurs; the volume of distribution during pseudoequilibrium (V_{area}), which occurs when net drug distribution between the plasma and the tissues reaches equilibrium and any decrease in plasma drug concentration is solely due to irreversible drug elimination from the body; and the

steady-state volume of distribution (V_{ss}), which is used under equilibrium conditions where plasma drug CL is null, such as during an i.v. infusion or once steady-state plasma concentrations are achieved following multiple drug administration (Toutain & Bousquet-Mélou, 2004b). Between drug administration (when V_c is calculated) and the attainment of distribution pseudo-equilibrium conditions (when V_{area} is calculated), V_d increases from V_c to V_{area} as the plasma drug concentration decreases due to distribution from the systemic circulation into the tissues. Thus, V_d during the phase of drug distribution is a time-dependent variable ranging from V_c to V_{area} (Toutain & Bousquet-Mélou, 2004b). If a large proportion of drug is eliminated from the body prior to attainment of distribution pseudo-equilibrium, then V_{area} may be much larger than V_{ss} . In most cases, however, V_{area} is only marginally larger than V_{ss} and will approach V_{ss} in cases where CL approaches zero (Toutain & Bousquet-Mélou, 2004b). Of the three V_d terms, V_{ss} is the most robust as it is independent of elimination (Baggot, 2006). In clinical practice, V_c is rarely used, although it may be useful for prediction of the initial maximum concentration of an i.v. bolus administration of a drug. V_{area} may be used to estimate the residual amount of drug in the body during elimination. V_{ss} holds the most clinical relevance, as it enables calculation of a loading dose (Toutain & Bousquet-Mélou, 2004b). Although V_d allows estimation of the extent of drug distribution within the body, it does not reveal the pattern of drug distribution or allow prediction of drug concentrations in tissues or at the site of infection (Baggot, 2006).

1.10.7 Terminal half-life

The plasma terminal half-life ($t_{1/2}$) is a composite pharmacokinetic parameter that is determined by systemic CL and V_d . The terminal $t_{1/2}$ is the time required for the plasma drug concentration to halve during the terminal phase (Toutain & Bousquet-Mélou,

2004c; Baggot, 2006). It is calculated using the elimination rate constant (k_{el}), which is the negative gradient of the terminal phase of the semi-logarithmic concentration-time curve (Riviere, 2009b). In some cases, the rate of drug absorption is slow enough to limit the rate of elimination. In these situations, a drug can only be eliminated as quickly as it is being absorbed, and the $t_{1/2}$ is reflective of the absorption rate rather than the elimination rate. This is known as absorption rate-limited disposition, or ‘flip-flop’ pharmacokinetics (Figure 1-4) (Toutain & Bousquet-Mélou, 2004c; Yáñez et al., 2011), and in this situation the terminal rate constant is an absorption constant, not an elimination constant; as such, it is termed k_a (Riviere, 2009b).

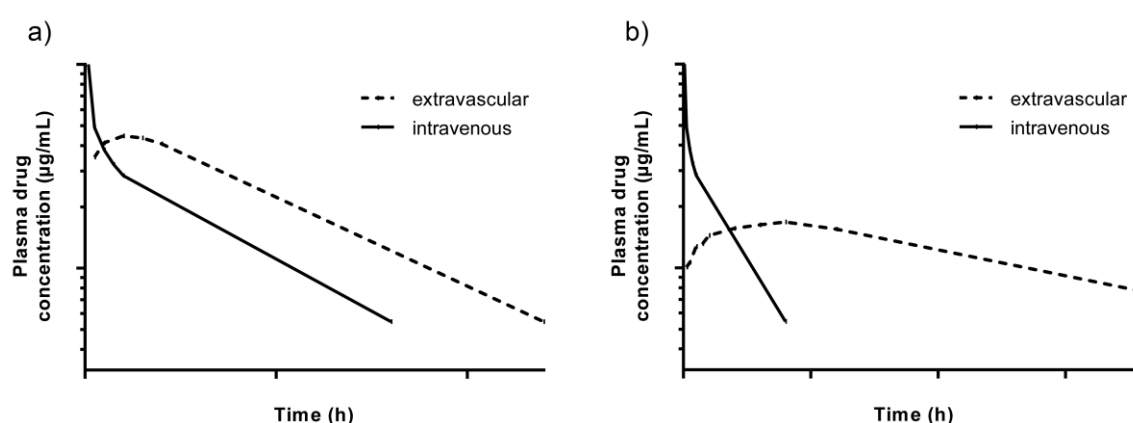


Figure 1-4. Theoretical concentration-time curves for a) a drug that does not display absorption rate-limited disposition, and b) a drug that does display absorption rate-limited disposition. In a), the similarity in gradient between both curves indicates a consistent rate of drug elimination, regardless of administration route. In b), the steeper gradient of the intravenous curve indicates the drug is being eliminated more slowly following extravascular administration, which is due to delayed absorption following this route of administration. Thus, the curves in b) depict absorption rate-limited disposition, or ‘flip-flop’ kinetics. Author’s own image.

Terminal $t_{1/2}$ is commonly used to compare the rate of drug elimination between species, and can vary dramatically for drugs that undergo hepatic metabolism (Baggot, 2006). Clinically, the terminal $t_{1/2}$ is used as a basis for selecting dosing intervals. Additionally, this parameter enables prediction of drug accumulation following multiple dosing and the time to reach steady state conditions (i.e. where the rate of drug input is

equal to the rate of drug output) (Toutain & Bousquet-Mélou, 2004c; Baggot, 2006), as well as the length of time it will take to eliminate the drug from the body following administration of the final dose (Riviere, 2009b).

Species differences in any of the processes of absorption, distribution, metabolism and elimination can lead to vast differences in the time course of drug molecules in the body (Riviere, 2009a), which can in turn have dramatic effects on drug pharmacodynamics.

1.10.8 Non-compartmental pharmacokinetic analysis

Non-compartmental pharmacokinetic analysis, utilising statistical moment theory, is frequently used to characterise drug pharmacokinetics, as it has the advantage of avoiding the assumptions required for specific compartmental models (Gabrielsson & Weiner, 2000). In addition to estimation of the CL, V_d , $t_{1/2}$, and k_{el} , important outcomes of non-compartmental analysis include measurement of the area under the concentration-time curve (AUC), which describes the degree of systemic drug exposure; the maximal concentration attained (C_{max}) and the time the C_{max} is reached (t_{max}), which are indicative of the drug absorption rate; and the mean residence time (MRT), which indicates the average time drug molecules stay in the body following administration (Baggot, 2006; Bulitta & Holford, 2008a). In statistical moment theory, the MRT is analogous to the terminal $t_{1/2}$, and is dependent upon the administration route. The MRT is estimated based on the AUC and the area under the first moment curve (AUMC). In the same way as the AUC is determined as the area under the curve when plasma concentration is plotted against time, the AUMC is determined as the area under the curve when the product of plasma concentration and time are plotted against time (Baggot, 2006).

1.10.9 Population pharmacokinetic modelling

Within any population, there is inherent inter-individual variability in drug pharmacokinetics following administration of a given drug dose (Drusano et al., 2001; Drusano, 2004). In comparison with non-compartmental analysis, population pharmacokinetic models are developed using probability distributions and have the advantage of being able to statistically account for this often large degree of between subject variability (BSV). Including BSV in the population pharmacokinetic model enables prediction of the range of drug concentrations and therapeutic responses anticipated for an entire patient population. Additionally, population pharmacokinetic models can be applied to sparse data sets and allow incorporation of multiple covariates, such as renal function, age, and body size into the model. This enables a quantitative assessment of the effect these factors may have on drug disposition (Bulitta & Holford, 2008b; Martín-Jiménez & Riviere, 1998).

1.10.10 Pharmacokinetic/pharmacodynamic indices

For anti-infective drugs, pharmacokinetic parameters can be combined with previously determined pharmacodynamic parameters, such as the minimum inhibitory concentration (MIC) of the target organism. The integration of these parameters enables estimation of a dosage that theoretically should reach a predetermined therapeutic target (Toutain & Lees, 2004). These therapeutic targets are considered PK/PD indices as they incorporate both a pharmacokinetic parameter and a pharmacodynamic parameter (Toutain et al., 2002). It was in the 1940s and 1950s that the effect of dosing frequency on antimicrobial efficacy was investigated, with variations in the penicillin administration schedule noted to have a significant effect on treatment outcome (Jawetz, 1946; Eagle et al., 1950). Successful therapy with penicillin was found to be dependent

upon the aggregate time for which plasma concentrations exceeded the bactericidal concentration, with no added benefit seen by further increasing the concentration (Eagle, 1948; Eagle et al., 1950; Eagle et al., 1953). This was recognised to differ from the pattern of activity observed with other antimicrobials available at the time, for which a positive correlation between concentration and efficacy seemed to exist, or that seemed to require a substantial host response to enable therapeutic efficacy (Eagle et al., 1953). Since these early pharmacodynamic studies, three PK/PD indices have been recognised, and they tend to vary between different antimicrobial classes. These are the AUC_{0-24}/MIC ratio, the C_{max}/MIC ratio, and the time for which the plasma concentration exceeds the MIC ($T > MIC$) (Toutain & Lees, 2004; Ambrose et al., 2007).

Population PK/PD modelling differs from PK/PD integration in that it involves computer simulations of pharmacokinetic and pharmacodynamic data. These data have usually been collected following administration of a single drug dose (Toutain & Lees, 2004). This provides a basis upon which to optimise dosage regimens based upon PK/PD indices. Estimated population pharmacokinetic parameters can be used in stochastic simulations, such as Monte Carlo simulations (MCS). MCS allow the relevant pharmacokinetic parameter to be estimated for each simulated subject (eg AUC , C_{max}), and these can then be integrated with the MIC data for the target organism. From this data, the probability of attaining the therapeutic target at various drug dosages can be calculated, thus providing an efficient and comprehensible method of enabling informed clinical decisions based upon PK/PD datasets (Drusano et al., 2001).

1.10.11 *In vitro* susceptibility testing

The MIC values used for PK/PD integration and PK/PD modelling are derived from *in vitro* antimicrobial susceptibility tests, and are specific for the microbe-antimicrobial combination tested. The MIC is defined as the minimum antimicrobial concentration required to inhibit growth of the microorganism being tested, whereas the minimum bactericidal concentration (MBC) is defined as the minimum concentration of antimicrobial required to prevent growth of a microorganism following subculture onto antimicrobial-free media (Andrews, 2001). *In vitro* susceptibility testing is usually undertaken according to standardised guidelines, to minimise the variability between operators and laboratories. Such guidelines are constructed by organisations such as the Clinical Laboratory Standards Institute (CLSI), and have been developed for many pathogens. However, there are no such guidelines for the *in vitro* susceptibility testing of *Chlamydia* spp., despite *C. trachomatis* being estimated to be the most prevalent bacterial sexually transmitted disease of humans by the World Health Organisation in 2005 (World Health Organization, 2011). Further to the lack of standardised guidelines for antimicrobial susceptibility testing of *Chlamydia* spp., there is only one paper reporting the antimicrobial susceptibility of *C. pecorum* isolates (Pudjiatmoko et al., 1998). These were isolated from ruminants, and did not involve testing of chloramphenicol, enrofloxacin or florfenicol.

1.11 DIETARY AND METABOLIC FACTORS THAT MAY INFLUENCE DRUG PHARMACOKINETIC PROFILES IN KOALAS

The finding of atypical pharmacokinetic profiles for some drugs in the koala is not unexpected. Although a number of suggested therapeutic drug dosages for koalas have been published without the benefit of pharmacokinetic studies (Blanshard, 1994; Booth

& Blanshard, 1999; Blanshard & Bodley, 2008), it has been recognised for over a decade that some drugs may be rapidly eliminated by koalas, and that dose extrapolation by allometric scaling may be inappropriate in this species (Booth & Blanshard, 1999). These insightful speculations were based upon findings of enhanced hepatic metabolic activity, as demonstrated by the rapid rate of bromsulphthalein clearance in koalas compared with sheep and macropods (Pass & Brown, 1990).

Heightened metabolic function of the koala liver is likely to have developed as an adaptation to the *Eucalyptus* spp. diet on which koalas are almost entirely dependent. This specialist diet is high in toxic plant secondary metabolites (PSMs), including phenolics, terpenes, and tannins (Cork & Foley, 1997; Pass et al., 1999), and only a fraction of the amount of *Eucalyptus* oil ingested daily by these folivorous marsupials is required to induce toxicity in humans after adjusting for body weight (Pass et al., 2001). Increased efficiency of certain metabolic pathways has been suggested as one method by which specialist folivores, including koalas, detoxify their diet (McLean & Foley, 1997; Boyle et al., 2000b; Boyle et al., 2001; Pass et al., 2002). There is evidence that certain Phase I oxidative reactions, such as those governed by CYPs, are very efficient in koalas, and have a high capacity (McLean & Foley, 1997; Boyle et al., 2001); this has been supported by *in vitro* assays using microsomes isolated from koala livers (Pass et al., 2001), and is intuitive for potentially toxic dietary components as extensive oxidation yields highly polar metabolites that can be excreted readily by the kidneys (Boyle et al., 2000b). Although Phase II glucuronidation reactions do occur in koalas, they appear less important than Phase I oxidation reactions in metabolising certain toxic dietary constituents (McLean & Foley, 1997). This pattern has also been observed in two other specialist feeders, the greater glider (*Petauroides volans*) and the ringtail

possum (*Pseudocheirus peregrinus*), and is in direct contrast with the metabolism of the same dietary constituents by a generalist feeder, the brushtail possum (*Trichosurus vulpecula*), which relies more heavily on Phase II glucuronidation for metabolism of these compounds (Boyle et al., 1999; Boyle et al., 2000a; Boyle et al., 2000b; Boyle et al., 2001). Additionally, the koala CYP responsible for tolbutamide hydroxylation, which is a known substrate for CYP2C in humans, shows a high affinity for tolbutamide and displays approximately ten-fold higher activity in koalas than in rats (Liapis et al., 2000). Highly efficient oxidative metabolism is likely responsible for the rapid elimination of meloxicam by koalas (Kimble et al., 2013), however, the effect of such efficient metabolic pathways on other therapeutic drugs is currently unknown, and warrants investigation.

1.12 DRUGS STUDIED IN THIS THESIS

The focus of this thesis is the characterisation of the pharmacokinetic profiles of three commonly used antimicrobials in koalas. Chloramphenicol and enrofloxacin were selected for investigation as they are the first-line anti-chlamydial drugs currently used in koalas, and as there is evidence that the currently used dosages of these drugs may be unsuitable for treatment of chlamydiosis (Griffith, 2010; Griffith et al., 2010; Govendir et al., 2012). In order for theoretical pharmacodynamic assessments of chloramphenicol and enrofloxacin to be made, *in vitro* susceptibility testing of these antimicrobials against koala isolates of *C. pecorum* was also performed. This included the chloramphenicol derivative, florfenicol, as the commercially available formulation of chloramphenicol was withdrawn from the market at the time the susceptibility testing was starting.

Fluconazole was selected due to its pharmacokinetic profile in other species, which is favourable for treating cryptococcosis (Humphrey et al., 1985). This profile consists of a long terminal $t_{1/2}$, large V_d , high bioavailability, and a low degree of protein binding, which allows penetration into the cerebrospinal fluid (CSF).

1.12.1 Chloramphenicol

Chloramphenicol is a broad-spectrum antimicrobial, with bacteriostatic activity against many gram negative and gram positive aerobes and anaerobes, as well as *Rickettsia* spp. and *Chlamydia* spp. (Dowling, 2006). Chloramphenicol use was first associated with fatal aplastic anaemia in humans in 1950 (Rich et al., 1950); as such, its systemic use in human medicine in Australia is now rare (Shultz et al., 2003), and off-label use in food-producing animals, including horses, is banned in Australia (Australian Veterinary Association, 2005). However, fatal aplastic anaemia has not been documented in domestic animals and the most notable adverse effect is dose-dependent bone marrow suppression affecting the erythroid precursor cells, which is reversible upon drug withdrawal (Papich & Riviere, 2009a). As a highly lipid soluble drug with moderately low protein-binding, chloramphenicol reaches effective concentrations in most body fluids and tissues, as well as in the CSF and central nervous system (CNS) (Dowling, 2006). Its high lipophilicity also enables penetration of cellular membranes, thus it has been used extensively to treat intracellular infections (Papich & Riviere, 2009a). Most studies that have investigated chloramphenicol pharmacokinetics in veterinary species have used colorimetry to determine plasma concentrations. This methodology is older than the chromatographic separation used in this study, and may make it difficult to draw solid conclusions regarding pharmacokinetic comparisons between studies. With this limitation in mind, the elimination $t_{1/2}$ of chloramphenicol varies between species

[0.98 h in horses, colorimetry (Sisodia et al., 1975); 1.3 h in pigs, colorimetry (Davis et al., 1972); 1.7 h in sheep, HPLC (Dagorn et al., 1990); 2.0 h in goats, colorimetry (Etuk et al., 2005); 4.2 h in dogs and 5.1 h in cats, colorimetry (Davis et al., 1972)]. Metabolism tends to be via Phase II conjugation with glucuronic acid, with inactive metabolites predominantly excreted in the urine (Papich & Riviere, 2009a). As chloramphenicol is not metabolised appreciably by Phase I oxidation, an investigation of its pharmacokinetic profile in healthy koalas may provide some information regarding the glucuronidation capacity of the koala's liver, as well as further insight into the suitability of the currently used chloramphenicol dosage regimen in koalas.

1.12.2 Enrofloxacin

Enrofloxacin is a fluoroquinolone antimicrobial with a broad spectrum of bactericidal aerobic activity, which includes *Chlamydia* spp. (Walker & Dowling, 2006; Kumar et al., 2007). This second generation quinolone is highly lipophilic, allowing it to penetrate into cells (Papich & Riviere, 2009b) and making it a good choice for treating intracellular bacteria. Following administration, enrofloxacin undergoes N-deethylation in the liver to form ciprofloxacin, an active metabolite (Papich & Riviere, 2009b). In some species the metabolism of enrofloxacin to ciprofloxacin is extensive, estimated as 20 to 35% horses (Kaartinen et al., 1997), 40% in dogs (Cester & Toutain, 1997) and 60% in cattle (Idowu et al., 2010); the two substances have an additive effect against some bacteria *in vitro* (Lautzenhiser et al., 2001), thereby potentially increasing the efficacy of enrofloxacin against susceptible bacteria *in vivo*. The subcutaneous and intramuscular bioavailability of fluoroquinolones are generally high, and despite having a short terminal $t_{1/2}$, enrofloxacin is reportedly effective when administered s.i.d. or b.i.d. (Paradis et al., 1990; Koch & Peters, 1996). One concerning factor associated with

fluoroquinolone use is the emergence of resistance, which is a recognised problem with the increasing use of this antimicrobial class in both humans and veterinary species (Endtz et al., 1991; Martin Barrasa et al., 2000; Hooper, 2001; Cohn et al., 2003). This, coupled with the current evidence that 10 mg/kg s.c. s.i.d. is ineffective at clearing *Chlamydia* spp. from koalas (Griffith, 2010), necessitates a pharmacokinetic evaluation of enrofloxacin following i.v. administration in order to establish a potentially efficacious dose rate.

1.12.3 Fluconazole

Fluconazole is a triazole antifungal drug used in both veterinary and human medicine. It has a broad spectrum of antifungal activity, which includes the *Cryptococcus neoformans* species complex (Giguère, 2006a). Fluconazole displays a vastly different pharmacokinetic profile from the other azole antifungals. It has almost complete oral bioavailability in many species (Humphrey et al., 1985; Brammer et al., 1990; Craig et al., 1994; Latimer et al., 2001; Davis et al., 2009), and its low molecular weight, large V_d , and low protein binding of 10 – 12% in most species (Humphrey et al., 1985) make it a good treatment option for CNS cryptococcosis, as it can be administered orally and reaches high concentrations in the CSF and most bodily fluids (Foulds et al., 1988; Walsh et al., 1989; Vaden et al., 1997; Latimer et al., 2001). The extended terminal $t_{1/2}$ [13 h in dogs (Humphrey et al., 1985); 14 - 25 h in cats (Craig et al., 1994; Vaden et al., 1997); 30 h in humans (Brammer et al., 1990); 41.6 h in horses (Latimer et al., 2001)] allows for infrequent administration, which is an important consideration when treating wildlife. Fluconazole undergoes minimal metabolism, being predominantly excreted unchanged in the urine (Giguère, 2006a). These factors should make it an ideal candidate for use in koalas, however, a vast range of dosages are currently used

(Blanshard & Bodley, 2008; Wynne et al., 2012), and no pharmacokinetic data are available for koalas.

1.13 AIMS OF THE THESIS

Drawing on the limited available information regarding drug absorption and elimination in koalas (Griffith et al., 2010; Govendir et al., 2012; Kimble et al., 2013), it is hypothesised that those therapeutic drugs that are subjected to hepatic metabolism will be rapidly metabolised and eliminated by koalas, and that many commonly used drugs will be poorly absorbed when administered orally. Consequently, it is hypothesised that the resulting plasma concentrations will be below theoretical therapeutic targets. In order to test these hypotheses, the overarching aim of this thesis is to characterise the pharmacokinetics of chloramphenicol, enrofloxacin and fluconazole in koalas, and to compare these profiles with those previously determined in other, physiologically distinct, species. When this study commenced, these three drugs were all first-line therapeutic options for koalas. Importantly, they are all known to undergo different routes of metabolism and elimination in other species; enrofloxacin and chloramphenicol are metabolised by Phase I (N-deethylation) and Phase II (glucuronidation) reactions, respectively, whereas fluconazole is almost exclusively eliminated by the kidneys. It was hoped that studying drugs that are metabolised and eliminated by differing routes would enable a rudimentary assessment of the efficiency of these metabolism and elimination pathways in this specialist folivore.

Aim 1: To characterise the pharmacokinetic profiles of the two injectable formulations of chloramphenicol available at the time of investigation following subcutaneous and

intravenous administration, in order to assess the likely suitability of the currently used dosing regimen of 60 mg/kg s.c. s.i.d.

Aim 2: To characterise the pharmacokinetics of enrofloxacin following i.v. administration to koalas in order to build upon the already existing subcutaneous and oral pharmacokinetic data, and to use this information to assess the likely suitability of enrofloxacin as a treatment option for chlamydiosis and other bacterial infections in koalas.

Aim 3: To characterise the pharmacokinetics of oral and i.v. fluconazole in koalas, to enable assessment of the likely suitability of fluconazole as a stand-alone drug for the treatment of koalas with cryptococcosis.

Aim 4: To determine the antimicrobial susceptibility of koala isolates of *C. pecorum* to enrofloxacin, chloramphenicol, and the chloramphenicol-derivative, florfenicol, and to integrate these results with pharmacokinetic parameters to enable an assessment of current dosing regimens to be made.

CONFIRMATION OF CO-AUTHORSHIP OF PUBLISHED WORK

I, Lisa Black, led the study design, data collection and analysis, and writing of this publication entitled 'Pharmacokinetics of chloramphenicol following administration of intravenous and subcutaneous chloramphenicol sodium succinate, and subcutaneous chloramphenicol, to koalas (*Phascolarctos cinereus*)'.

Andrew McLachlan provided advice regarding the pharmacokinetic analysis, and assistance finalising the manuscript prior to publication. Joanna Griffith provided advice regarding study design and execution, and assistance finalising the manuscript prior to publication. Merran Govendir, Mark Krockenberger, and Damien Higgins all assisted in the design of the project and provided assistance finalising the manuscript prior to publication. Amber Gillett assisted with getting ethical approval and coordinating field work, and provided assistance with finalising the manuscript prior to publication.

Lisa Black



Date 31/7/2014

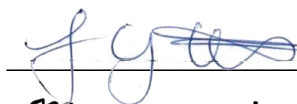
I, as co-author, endorse that this level of contribution by myself and the candidate indicated above is appropriate:

Andrew McLachlan



Date 8/11/2013

Joanna Griffith



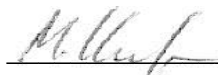
Date 1/11/2013

Merran Govendir



Date 31/7/2014

Mark Krockenberger



Date 31/7/2014

Damien Higgins



Date 31/7/2014

Amber Gillett



Date 26/10/2013

CHAPTER 2. PHARMACOKINETICS OF CHLORAMPHENICOL FOLLOWING ADMINISTRATION OF INTRAVENOUS AND SUBCUTANEOUS CHLORAMPHENICOL SODIUM SUCCINATE, AND SUBCUTANEOUS CHLORAMPHENICOL, TO KOALAS (*PHASCOLARCTOS CINEREUS*)

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Black, L.A., McLachlan, A.J., Griffith, J.E., Higgins, D.P., Gillett, A., Krockenberger, M.B. & Govendir, M. (2012) Pharmacokinetics of chloramphenicol following administration of intravenous and subcutaneous chloramphenicol sodium succinate, and subcutaneous chloramphenicol, to koalas (*Phascolarctos cinereus*). *Journal of Veterinary Pharmacology and Therapeutics*, **36**, 478-485.

2.1 ABSTRACT

Clinically normal koalas (n = 19) received a single dose of intravenous chloramphenicol sodium succinate (SS) (25 mg/kg; n = 6), subcutaneous chloramphenicol SS (60 mg/kg; n = 7) or subcutaneous chloramphenicol base (60 mg/kg; n = 6). Serial plasma samples were collected over 24 - 48 h and chloramphenicol concentrations determined using a validated HPLC assay. The median (range) apparent clearance (CL/F) and elimination half-life ($t_{1/2}$) of chloramphenicol after intravenous chloramphenicol SS administration were 0.52 (0.35 - 0.99) L/h/kg and 1.13 (0.76 – 1.40) h, respectively. Although the area under the concentration-time curve was comparable for the two subcutaneous

formulations, the absorption rate-limited disposition of chloramphenicol base resulted in a lower median $C_{\max}$ (2.52; range 0.75 – 6.80 $\mu\text{g/mL}$) and longer median $t_{\max}$ (8.00; range 4.00 – 12.00 h) than chloramphenicol SS ($C_{\max}$ 20.37, range 13.88 – 25.15 $\mu\text{g/mL}$; $t_{\max}$ 1.25, range 1.00 – 2.00 h). When these results were compared with susceptibility data for human *Chlamydia* isolates, the expected efficacy of the current chloramphenicol dosing regimen used in koalas to treat chlamydiosis remains uncertain, and at odds with clinical observations.

2.2 INTRODUCTION

The koala (*Phascolarctos cinereus*) is an Australian icon of both ecological and economic significance. Currently, koalas are listed as vulnerable in some Australian states, with two populations listed as endangered (Department of the Environment, Water, Heritage and the Arts, 2009). Chlamydiosis is the most common infectious disease in some koala populations (Stalder, 2003; Loader, 2010) and may cause high morbidity or even mortality, resulting from one or more of the following: keratoconjunctivitis (Cockram & Jackson, 1976), a rhinitis/pneumonia complex (Nicolson, 2002) and urogenital inflammation and fibrosis (Brown & Grice, 1986) which can cause infertility. Chlamydiosis is caused by the obligate intracellular bacteria *Chlamydia pecorum* and *C. pneumoniae*, with the former being the more pathogenic of the two in koalas (Jackson et al., 1999).

Chloramphenicol is one of the few systemic antimicrobials currently used for treating koalas with chlamydiosis due to the syndrome of inappetence, wasting and death that is induced by macrolides and tetracyclines (Brown et al., 1984; Osawa & Carrick, 1990). Enrofloxacin is also used to treat koala chlamydiosis, but it does not prevent

recrudescence of infection after a prolonged course of treatment (Griffith, 2010). Chloramphenicol appears to be efficacious, as evidenced by the absence of chlamydial shedding during the post-treatment monitoring phase [two weeks in one study (Markey et al., 2007) and up to 63 days in another (Govendir et al., 2012)].

As chloramphenicol exhibits a lack of correlation between pharmacokinetic parameters and interspecies body size when subjected to allometric scaling (Riviere et al., 1997), it is unsuitable for interspecies dose extrapolation. Despite this, and its routine use in some wildlife facilities (Blanshard & Bodley, 2008), there is limited pharmacokinetic data available regarding chloramphenicol in koalas. Currently, some pharmacokinetic data exist for chloramphenicol base administered to diseased koalas only (Govendir et al., 2012) and no intravenous (i.v.) data have been reported. The current study aimed to determine pharmacokinetic parameters for chloramphenicol following administration of two formulations to healthy koalas. The inactive prodrug, chloramphenicol sodium succinate (SS), which is hydrolysed to biologically active chloramphenicol following administration, was selected as it is the only i.v. formulation available in Australia. Subcutaneous (s.c.) pharmacokinetics were compared between chloramphenicol SS and the commonly used, veterinary registered chloramphenicol base formulation. The results were compared to susceptibility data for human *C. trachomatis* as a means to estimate the suitability of the currently recommended dosing regimen of 60 mg/kg s.c. once daily (s.i.d.) (Blanshard & Bodley, 2008) in koalas.

2.3 MATERIALS AND METHODS

2.3.1 Animals

Clinically normal subadult and adult koalas (n = 19; 7 males, 12 females), ranging in age from 15 months to seven years, as determined by tooth wear (Martin, 1981) or previous admission data, were recruited opportunistically from those housed temporarily at the Australia Zoo Wildlife Hospital (AZWH), Beerwah, Queensland, Australia. Koalas had initially been hospitalised for injury or illness, or presented as orphaned joeys and provided sanctuary while they matured. All koalas were being prepared for release back into the wild and were deemed healthy prior to the study, based on results of a full physical examination conducted under general anaesthesia by veterinarians at AZWH, and routine haematology and biochemistry analytes within reference ranges (Canfield et al., 1989; Blanshard, 1994). Koalas were housed in standard veterinary hospital enclosures, and supplied with food *ad libitum* (various *Eucalyptus* spp.) and water throughout the study. This study was approved by The University of Sydney Animal Ethics Committee (protocol number N00/10-2007/4/4695).

General anaesthesia was induced by mask using 3 – 5% isoflurane in 100% oxygen delivered by a precision vaporiser and maintained for 5 - 10 min, to enable placement of a 20-gauge i.v. catheter into the cephalic vein for serial blood sampling. Koalas were then recovered for one to two hours before drug was administered at t = 0.

Koalas were divided into three treatment groups: i.v. chloramphenicol SS (n = 6), s.c. chloramphenicol SS (n = 7) and s.c. chloramphenicol base (n = 6). The i.v. group received a 1 min i.v. infusion of chloramphenicol SS (Kemicetine® Succinate Injection,

Actavis Italy, Milan, Italy) equivalent to 25 mg/kg chloramphenicol, diluted 1:5 in sterile water for injections. The s.c. groups received chloramphenicol SS or chloramphenicol base (Chloramphenicol 150, Delvet, Seven Hills, NSW, Australia) at 60 mg/kg as a single s.c. injection. Serial blood samples (up to 1.5 mL) for chloramphenicol concentration determination were collected into lithium heparin tubes at the following time points: t = 0, 2, 15, 30, 45 min, 1, 1.5, 2, 4, 6, 8, 12 and 24 h for the i.v. group; and t = 0, 15, 30 min, then 1, 1.5, 2, 4, 6, 8, 12, 24, 36 and 48 h for both s.c. groups. Blood samples were centrifuged within 1 h of collection and plasma was transferred into plain tubes. Samples were stored at -20 °C and protected from light until analysis.

2.3.2 Analytical method

Plasma concentrations of chloramphenicol were determined using a reversed-phase high-performance liquid chromatography (HPLC) method described previously (Govendir et al., 2012), using an SPD-M20A diode array detector (Shimadzu, Rydalmere, NSW, Australia) set at 270 nm. Data acquisition and peak integration were performed by Shimadzu Class VP software, version 7.4. Retention times for chloramphenicol and the internal standard (IS) were 5.62 and 6.41 min, respectively. Pooled, drug-free koala plasma exhibited no interfering peaks at the retention times of interest.

Triplicate 200 µL samples of medicated koala plasma were analysed. Calibration standards (0.1, 0.5, 1, 5, 10, 50, 100, 500 µg/mL) were prepared daily using pooled drug-free koala plasma to prepare serial dilutions of an 8,000 µg/mL chloramphenicol stock solution (analytical grade chloramphenicol, Sigma-Aldrich, Castle Hill, NSW,

Australia) dissolved in analytical grade methanol (Lomb Scientific, Taren Point, NSW, Australia). Prior to solid phase extraction (SPE), 50 μL of 50 $\mu\text{g/mL}$ phenacetin (Sigma-Aldrich, Castle Hill, NSW, Australia) was added to calibration standards and plasma samples as the IS.

Triplicate plasma samples and calibration standards underwent SPE using an Oasis HLB 1 cm^3 column filter (Waters, Milford, Massachusetts, USA) via a vacuum manifold, as per manufacturer's instructions. Eluates were evaporated to dryness at 35 $^{\circ}\text{C}$ for 3 h using a vacuum concentrator (SPD 121P, Thermo Electron Corporation, Asheville, North Carolina, USA), and reconstituted using 100 μL mobile phase, which consisted of acetonitrile (Lomb Scientific, Taren Point, NSW, Australia) and deionised water (40:60, v/v). Samples were vortexed for 10 s, and centrifuged for 10 min at 14,000 $\times g$. The supernatant was injected into the HPLC system in duplicate.

Chloramphenicol recovery was $86 \pm 0.08\%$, determined by comparing replicates of three known chloramphenicol concentrations spanning the calibration range spiked into blank koala plasma prior to, and following, SPE.

Interpolation of the daily calibration curve with a minimum coefficient of determination (R^2) > 0.999 was used to determine the concentration of chloramphenicol in treated koala plasma. A lack-of-fit test was also performed using GenStat 14th edition (VSN International, 2011) to verify linearity of the calibration curve, and showed no deviation from the linear model ($F = 0.42$; d.f. = 6, 69; $P = 0.87$). The lower limit of detection (LLD) and lower limit of quantification (LLQ) were calculated at 0.07 $\mu\text{g/mL}$ and 0.20 $\mu\text{g/mL}$ respectively, using the standard deviation of the y-intercepts of the regression

line (International Conference on Harmonisation, 2005). Intra-assay variation was assessed by the comparison of six replicates of five calibration standards (1, 5, 10, 50, 100 µg/mL), assayed on a single day. Inter-assay variation was assessed by comparing replicates of three calibration standards (1, 10, 100 µg/mL) on three analysis days. Intra- and inter-assay variation were < 8.71% and < 12.25%, respectively.

2.3.3 Plasma protein binding

The proportion of chloramphenicol binding to plasma proteins was determined by ultrafiltration (Dow, 2006). The plasma pH and protein concentration of freshly collected, pooled drug-free plasma from eight koalas were determined prior to the assay. Analytical grade chloramphenicol was added to 1 mL plasma aliquots to yield concentrations of 0.5, 50 and 500 µg/mL. Samples were incubated at 36 °C to mimic koala core body temperature (Degabriele & Dawson, 1979) for 30 min, then centrifuged (4000 x g for 30 min) in a fixed angle rotor centrifuge (Eppendorf 5417C; Eppendorf, Hamburg, Germany) using an ultrafiltration tube (Amicon® Ultra-0.5 30 kDa, Merck Millipore, Kilsyth, Victoria, Australia). The non-protein bound ultrafiltrate was recovered and the chloramphenicol concentration determined. This concentration was compared with standards of the same concentrations made in mobile phase. In addition, non-specific binding to the ultrafiltration device was determined by repeating the above steps using phosphate buffered saline instead of plasma. All samples were prepared and analysed in triplicate. Percentage binding to plasma proteins was determined using the following equation:

$$\% \text{ protein binding} = 100 - \left(\frac{\text{unbound drug}}{\text{total drug}} \right) \times 100$$

2.3.4 Pharmacokinetic analysis

The data from each koala were analysed using a standard non-compartmental pharmacokinetic approach, for values above the LLQ. The maximal concentration ($C_{\max}$) and time to reach maximal concentration ($t_{\max}$) were determined graphically. The terminal elimination rate constant (k_{el}) was determined as the slope of the terminal portion of the natural log of the concentration-time curve. The terminal elimination rate constant was assumed to be the absorption rate constant (k_a) for chloramphenicol base as this formulation exhibited absorption rate-limited disposition (Yáñez et al., 2011). For SC chloramphenicol SS, k_a was determined using the method of residuals. The area under the concentration-time curve (AUC_{0-t}) and area under the first moment curve ($AUMC_{0-t}$) were calculated to the last measurable concentration using the linear trapezoidal method. The elimination half-life ($t_{1/2}$), apparent clearance (CL/F), apparent volume of distribution at steady state (V_{ss}/F), mean residence time (MRT), predicted average steady state plasma concentration ($C_{ss\text{average}}$), predicted unbound steady state plasma concentration ($C_{ssu\text{average}}$), bioavailability (F) and terminal segments of the $AUC_{0-\infty}$ and $AUMC_{0-\infty}$ ($AUC_{\text{extrapolated}}$ and $AUMC_{\text{extrapolated}}$ respectively) were calculated using the following equations:

$$t_{1/2} = \ln 2 / k_{el}$$

$$CL/F = \text{Dose} / AUC_{0-\infty}$$

$$V_{ss}/F = CL/F \times MRT$$

$$MRT = AUMC_{0-\infty} / AUC_{0-\infty}$$

$$C_{ss\text{average}} = AUC_{0-\infty} / \tau, \text{ where } \tau \text{ is the dosing interval (12 or 24 h).}$$

$$C_{ssu\text{average}} = C_{ss\text{average}} \times f_u, \text{ where } f_u \text{ is the unbound fraction in plasma (0.416).}$$

F = $(AUC_{sc} / AUC_{iv}) \times (Dose_{iv} / Dose_{sc})$, where AUC_{sc} is the individual $AUC_{0-\infty}$ term for each koala in the SC chloramphenicol SS treatment group, and AUC_{iv} is the mean of the $AUC_{0-\infty}$ data from the IV treatment group.

$AUC_{extrapolated} = C_{last} / k_{el}$, where C_{last} is the last measured plasma concentration.

$AUMC_{extrapolated} = [(C_{last} \times t_{last}) / k_{el}] + (C_{last} / k_{el}^2)$, where C_{last} is the last measured plasma concentration, and t_{last} is the time of the last measured plasma concentration.

Maintenance dose rate = target $Css_{(average)} \times CL/F$, where the target $Css_{(average)}$ is extrapolated from minimum inhibitory concentration (MIC) data.

$AUC_{extrapolated}$ was only included if it comprised < 20% of the $AUC_{0-\infty}$.

2.3.5 Statistical analysis

A Kruskal-Wallis analysis of variance, followed by a Mann-Whitney test where applicable, was used to compare the terminal rate constant and $t_{1/2}$ between the three treatment groups. A Mann-Whitney test was used to compare C_{max} , t_{max} , $AUC_{0-\infty}$, $AUMC_{0-\infty}$ and MRT between the two subcutaneous formulations; parameters for chloramphenicol base in healthy koalas with those determined previously in koalas with chlamydiosis (Govendir et al., 2012); and to assess for differences in pharmacokinetic parameters calculated for subadult versus adult koalas within treatment groups. Statistical significance for all tests was accepted at $P < 0.05$.

2.4 RESULTS

The median (and range) of calculated pharmacokinetic parameters are presented in Table 2-1. Binding of chloramphenicol to plasma proteins was $58.4 \pm 4.4\%$ ($n = 8$), and

non-specific binding to the ultrafiltration device was < 5%. The semi-logarithmic plasma drug concentration-time curves for each treatment group are presented in Figure 2-1. The $C_{\max}$ of biologically active chloramphenicol for all koalas in the IV group was recorded at $t = 2$ min. The i.v. chloramphenicol SS semi-logarithmic plasma drug concentration-time curve showed a biphasic decline, and distribution equilibrium was reached by $t = 15$ min. There was no quantifiable chloramphenicol in the plasma of any koala in the i.v. group after $t = 8$ h.

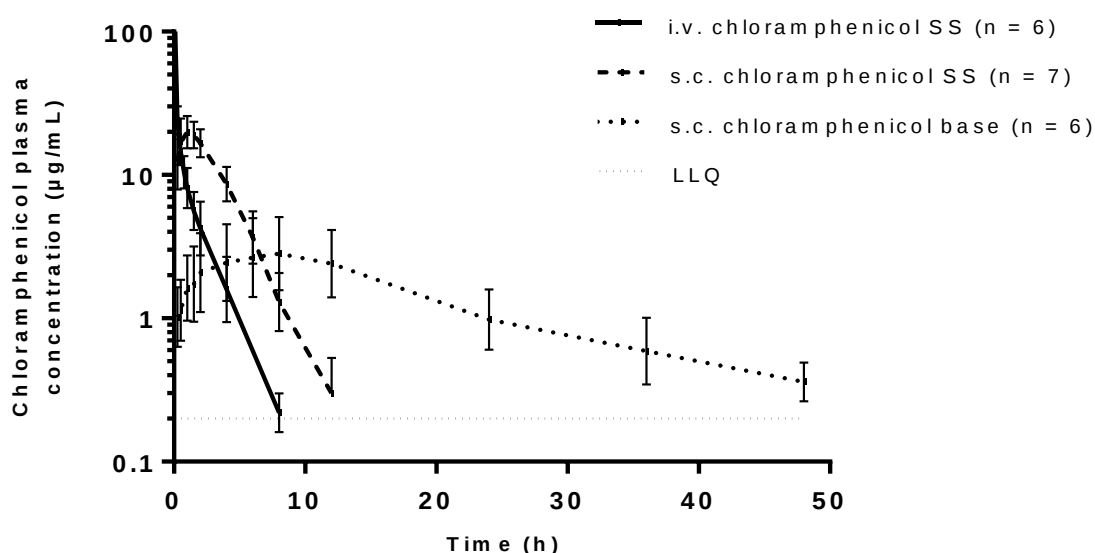


Figure 2-1. Semi-logarithmic plasma concentration-time curve (mean $\pm$ SD) for i.v. chloramphenicol SS (25 mg/kg), s.c. chloramphenicol SS (60 mg/kg) and s.c. chloramphenicol base (60 mg/kg).

Table 2-1. Pharmacokinetic parameters (median and range) estimated following administration of chloramphenicol sodium succinate and chloramphenicol base. i.v.: intravenous administration; s.c.: subcutaneous administration; s.i.d: once daily dosing; b.i.d: twice daily dosing. * denotes predicted concentrations following administration of 60 mg/kg s.c. § denotes calculations that have excluded two koalas from s.c. chloramphenicol base group whose $AUC_{\text{extrapolated}}$ was > 20% of the $AUC_{0-\infty}$. For the statistical analysis, # was compared with k_a for s.c. chloramphenicol base, as both were the terminal rate constants.

	Chloramphenicol sodium succinate i.v. (25 mg/kg, n = 6)	Chloramphenicol sodium succinate s.c. (60 mg/kg, n = 7)	Chloramphenicol Base s.c. (60 mg/kg, n = 6)	Chloramphenicol base s.c.; diseased koalas, day 1 (60mg/kg, n = 9) (Govendir et al., 2012)
C_{max} (µg/mL)	181.89 (109.78 – 252.41)	20.37 (13.88 – 25.15)	2.52 (0.75 – 6.80)	3.02 (1.32 – 5.03)
t_{max} (h)	-	1.25 (1.00 – 2.00)	8.00 (4.00 – 12.00)	4.00 (1.00 – 8.00)
k_{el} (h ⁻¹)	0.62 (0.50 – 0.91)	0.48 (0.36 – 0.66)	-	0.03 (0.02 – 0.11) [#]
k_a (h ⁻¹)	-	5.93 (4.55 – 6.49)	0.06 (0.02 – 0.09)	-
$t_{1/2}$ (h)	1.13 (0.76 – 1.40)	1.45 (1.05 – 1.93)	13.14 (7.47 – 35.39)	-
V_{ss}/F (L/kg)	0.45 (0.29 – 0.78)	-	-	-
CL/F (L/h/kg) [§]	0.52 (0.35 – 0.99)	-	0.85 (0.40 – 1.76)	-
AUC_{0-t} (µg/mL.h)	-	-	49.81 (27.25 – 143.12)	48.14 (22.37 – 81.14)
$AUMC_{0-t}$ (µg/mL.h ²)	-	-	645.43 (352.98 – 2078.94)	530.03 (233.05 – 798.97)
$AUC_{0-\infty}$ (µg/mL.h) [§]	48.41 (25.17 – 72.02)	75.60 (54.71 – 106.82)	70.34 (34.06 – 149.04)	-
MRT (h) [§]	0.90 (0.49 – 1.27)	2.66 (2.19 – 4.02)	14.40 (13.95 – 15.53)	-
F	-	0.68 (0.49 – 0.95)	-	-
$C_{\text{ssaverage}}$ (µg/mL)* [§] s.i.d dose	-	-	2.93 (1.42 – 6.21)	-
$C_{\text{ssuaverage}}$ (µg/mL)* [§] s.i.d dose	-	-	1.22 (0.59 – 2.58)	-
$C_{\text{ssaverage}}$ (µg/mL)* [§] b.i.d dose	-	-	5.86 (2.84 - 12.42)	-
$C_{\text{ssuaverage}}$ (µg/mL)* [§] b.i.d dose	-	-	2.44 (1.18 - 5.17)	-

Some of the pharmacokinetic parameters estimated for chloramphenicol base exhibited greater inter-individual variation than those for chloramphenicol SS. The $AUC_{\text{extrapolated}}$ for all animals in the s.c. chloramphenicol SS treatment group, and for four of the six animals in the chloramphenicol base treatment group, comprised $< 5.19\%$ of the $AUC_{0-\infty}$. The remaining two animals had an $AUC_{\text{extrapolated}} > 20\%$ (23.97% and 45.66%) of the $AUC_{0-\infty}$, and were subsequently excluded from any calculations involving the $AUC_{0-\infty}$. The final quantifiable chloramphenicol concentrations were detected in plasma drawn at $t = 8 \text{ h}$ ($n = 2$) and $t = 12 \text{ h}$ ($n = 5$) for s.c. chloramphenicol SS, and $t = 24 \text{ h}$ ($n = 1$), $t = 36 \text{ h}$ ($n = 2$) and $t = 48 \text{ h}$ ($n = 3$) for chloramphenicol base.

There were no statistically significant differences between values calculated for C_{max} , t_{max} , k_{el} , $AUMC_{0-t}$ or AUC_{0-t} in this study compared with those determined previously in koalas with chlamydiosis (Govendir et al., 2012), and no significant differences were found between adult and subadult koalas within the same treatment groups. The terminal rate constant was significantly lower and the $t_{1/2}$ significantly longer for s.c. chloramphenicol base compared with the two chloramphenicol SS treatment groups. Significant differences were found between the two subcutaneous groups for all other parameters, except the AUC. The demeanour, browsing habits and faecal output of each animal were unchanged during and following the study, and there were no apparent adverse effects resulting from drug administration, i.v. catheter placement, frequent handling or hospitalisation.

2.5 DISCUSSION

This is the first study to report the pharmacokinetic profiles of two different formulations of chloramphenicol in clinically normal koalas. The extended sampling

time and validated HPLC assay enabled characterisation of the entire single-dose pharmacokinetic curves for both chloramphenicol formulations for all but two koalas. Although the pharmacokinetics of chloramphenicol following administration of chloramphenicol SS documented here are similar to those reported in other species, this formulation reaches much higher plasma concentrations and is eliminated more quickly than chloramphenicol base, thereby potentially increasing the risk of toxicity and requiring more frequent dosing than the base formulation. An important finding was the observation of absorption rate-limited disposition of chloramphenicol base, which was responsible for the significant differences in the elimination rate between the two chloramphenicol formulations. This has important treatment implications, suggesting that chloramphenicol base may be the more suitable of the two formulations for subcutaneous use in koalas owing to the shape of the concentration-time curve, although the currently used dose appears inadequate.

As specialist folivores, koalas possess highly efficient hepatic metabolism to enable detoxification of their eucalyptus diet, which contains high concentrations of plant secondary metabolites such as phenols and terpenes (Cork & Foley, 1997); these compounds are toxic to most mammals. Augmented liver function has been demonstrated in koalas by an increased rate of bromsulphthalein clearance, relative to sheep and some macropods (Pass & Brown, 1990). Additionally, certain cytochrome P450 enzymes (CYPs) involved in xenobiotic metabolism are increased in quantity and capacity in koala livers, relative to generalist herbivores (Liapis et al., 2000; Ngo et al., 2000). Despite this, the elimination of chloramphenicol is similar between koalas and various other species. The $t_{1/2}$ and k_{el} of i.v. chloramphenicol in koalas are similar to those established for horses ($t_{1/2}$, 1 ± 0.1 h; k_{el} , 0.69 ± 0.1 h⁻¹) (Pilloud, 1973) while a

slightly longer $t_{1/2}$ has been observed in sheep (1.70 ± 0.02 h) (Dagorn et al., 1990) and goats (1.97 ± 1.23 h) (Etuk et al., 2005). Although the metabolic pathway of chloramphenicol has not been determined in koalas, chloramphenicol is metabolised by glucuronidation in other species (Papich & Riviere, 2009a). Hence the similar elimination rates observed between koalas, horses, sheep and goats, may indicate a similar glucuronidation capacity between these species. In contrast, the non-steroidal anti-inflammatory drug meloxicam, which undergoes Phase I metabolism via oxidation, is very rapidly eliminated from the plasma following administration to koalas (Kimble et al., 2013), possibly due to active biotransformation by CYPs as an adaptation to enable detoxification of their highly toxic eucalypt diet (Liapis et al., 2000). Oxidation has been postulated as being the most important mechanism for detoxification of dietary PSMs in specialist folivores such as koalas (Boyle et al., 1999), whereas Phase II conjugation reactions, including glucuronidation, appear to be involved minimally (McLean & Foley, 1997). This may have implications for selection of therapeutic drugs in koalas, as drugs that are metabolised by conjugation reactions may have a more favourable disposition in koalas than those metabolised by oxidation.

The pharmacodynamic surrogate most closely associated with chloramphenicol efficacy is the time for which plasma concentration exceeds the MIC of the target pathogen (Giguère, 2006b). Thus, the prolonged $t_{1/2}$ of chloramphenicol observed after s.c. administration of the base renders it a more appropriate formulation than chloramphenicol SS for targeting chlamydial infections in koalas, based on the shape of the concentration-time curve (Figure 2-2). The *in vitro* susceptibility of koala strains of *C. pecorum* and *C. pneumoniae* to chloramphenicol have not been established, but antibacterial susceptibility patterns appear uniform across chlamydial species

(Miyashita et al., 1997; Pudjiatmoko et al., 1998) and hosts (Kumar et al., 2007), including isolates of *C. pneumoniae* from another native Australian marsupial, the western barred bandicoot (*Perameles bougainville*) (Kumar et al., 2007). In the current study, theoretical chloramphenicol MICs for *C. pecorum* and *C. pneumoniae* were assumed, based on those established for *C. trachomatis*, a widely studied human pathogen, and ranged from 1 µg/mL (Johnson & Hobson, 1977; Hobson et al., 1982a; How et al., 1985; Bianchi et al., 1988) to 4 µg/mL (Ridgway et al., 1978; Cevenini et al., 1987). The predicted $C_{ssu_{average}}$ (median 1.22 µg/mL) of chloramphenicol base marginally exceeded 1 µg/mL in 50% of koalas in this study, but did not reach the higher target of 4 µg/mL in any koalas. However, the predicted $C_{ssu_{average}}$ for twice daily dosing exceeded 1 µg/mL in all koalas, indicating twice daily dosing may be more appropriate than once daily dosing, and warrants pharmacokinetic investigation. However, the authors warn that extreme caution should be exercised if pharmacokinetic investigations were to proceed following twice daily dosing, as doses of 80 mg/kg SC s.i.d. have been associated with fatal gastrointestinal dysbiosis in some koalas (A. Gillett 2011, pers. obs.)¹. For this reason, twice daily dosing at a lower dose may be more suitable for future investigations, however extreme caution and vigilance are still required due to the risks involved. The discrepancy between the subtherapeutic plasma concentrations attained and the observed reduction in chlamydial shedding during, and for a short time following, treatment (Markey et al., 2007; Govendir et al., 2012) at the current dose rate demonstrates the need for koala isolates of *C. pecorum* and *C. pneumoniae* to undergo *in vitro* susceptibility testing to verify therapeutic targets.

¹ Personal observation by one of the authors: Amber Gillett, Wildlife Veterinarian, Australia Zoo Wildlife Hospital, Qld, October 2011.

Although these tests will not necessarily reflect the situation *in vivo*, they can be a useful guide for dose development.

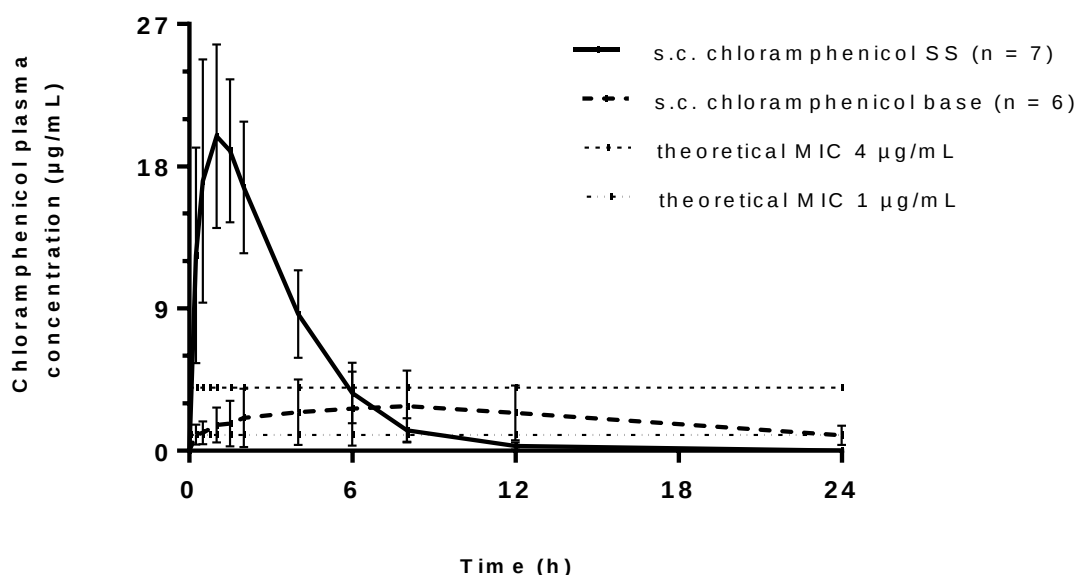


Figure 2-2. Plasma concentration-time curve (mean $\pm$ SD) for s.c. chloramphenicol SS (60 mg/kg) and s.c. chloramphenicol base (60 mg/kg).

Calculating a theoretical dose of chloramphenicol base to achieve therapeutic targets proved difficult due to the marked variation in pharmacokinetic profiles observed among koalas. Although this may in part be due to inter-individual variation, erratic absorption of this insoluble formulation is a likely cause of the variation between koalas. Theoretical doses of SC chloramphenicol base (95th percentile (median; range)) for once daily dosing, calculated to exceed MIC targets of 1 µg/mL and 4 µg/mL were 94 (49; 23 – 102) mg/kg and 375 (197; 93 – 407) mg/kg respectively; and for twice daily dosing were 47 (25; 12 – 51) mg/kg and 188 (98; 46 – 203) mg/kg respectively. The variability in proposed dose rates is likely due to the physicochemical nature of chloramphenicol and may be overcome to some extent by the choice and design of the chloramphenicol formulation, such as a formulation compounded into a sustained-

release vehicle, which may help to stabilise and solubilise this poorly soluble drug (Bonacucina et al., 2011). Such a formulation would presumably lead to a more uniform absorption profile, thereby making calculation of a standard dose more feasible. Alternatively, the chloramphenicol derivative, florfenicol, may have a more uniform pharmacokinetic profile between individuals and warrants investigation in this species. Florfenicol may also be desirable as the commercially available preparation exhibits absorption-rate limited disposition (Dowling, 2006), and it lacks the para-nitro group thought to be responsible for the fatal aplastic anaemia in humans that can occur after contact with chloramphenicol (Papich & Riviere, 2009a), potentially making it a safer option for those in contact with treated animals and their excrement.

Several factors may contribute to reduction in chlamydial shedding in the absence of target plasma concentrations. Firstly, intracellular concentrations of chloramphenicol are likely to exceed plasma concentrations (English & Withy, 1959; English & Seawright, 1961; Watson & McDonald, 1976; Etuk & Onyeyili, 2005). This is due to the high lipophilicity of chloramphenicol allowing it to cross the cell membrane readily, which can lead to extensive tissue penetration (Ambrose, 1984; Papich & Riviere, 2009a) and is an important drug characteristic when treating intracellular bacteria such as *Chlamydiae*. Secondly, chloramphenicol persists in various tissues (English & Withy, 1959; Watson & McDonald, 1976) and reaches high concentrations in the urine (Gruhzt et al., 1949; English & Withy, 1959) hours after the plasma concentrations have reached undetectable levels, implying that chloramphenicol is quickly redistributed into tissues where it persists for prolonged periods, before slowly being released back into the systemic circulation and excreted in the urine (Gruhzt et al., 1949). Finally, chloramphenicol has the ability to block the anti-apoptotic activity (Fan

et al., 1998) used by *Chlamydiae* to protect themselves from apoptosis of the host cell (Greene et al., 2004) in which they develop and replicate (Zhong & Brunham, 1998), and this may contribute to chlamydial killing by cytotoxic T cells and host cell defences.

A limitation of this study was the unknown rate and extent of hydrolysis of chloramphenicol SS to active chloramphenicol in koalas. Due to the instability of chloramphenicol SS in plasma (Nahata & Powell, 1981) and the remote location of the field site, samples were unable to be transported to the laboratory in time to enable analysis of the plasma concentration. The bioavailability of chloramphenicol following i.v. chloramphenicol SS administration is approximately 0.7 in humans (Glazko et al., 1977; Burke et al., 1982), 10 - 12 week old calves (Reiche et al., 1980) and adolescent pigtail macaques (Koup et al., 1981) as approximately 30% of the succinate ester is excreted unhydrolysed in the urine. This limitation may have led to an overestimation of chloramphenicol CL and V_d in the current study, which could only be estimated as apparent values (CL/F and V_{ss}/F , respectively). Despite this limitation, we can conclude that the clinical usefulness of chloramphenicol SS in koalas is limited by its rapid CL. Further, the estimated steady state plasma concentrations and subsequent dosing calculations are not affected by an overestimation of chloramphenicol SS CL or V_d , so this limitation is considered to be of little clinical consequence to this study.

This study demonstrated the pharmacokinetic properties of two chloramphenicol formulations in the koala. These results support chloramphenicol base as the more suitable treatment option of the two formulations studied, although the adequacy of the currently used dose remains questionable, and more appropriate plasma concentrations

may be attained following administration of a lower dose twice daily. The marked variation in the absorption rate of chloramphenicol base between individuals demonstrates the potential value in investigating alternative chloramphenicol formulations to achieve a more uniform absorption. Additionally, further work needs to be undertaken to determine the *in vitro* MIC for koala isolates of *C. pecorum* and *C. pneumoniae*, and to investigate the intracellular concentrations of chloramphenicol in koalas.

CONFIRMATION OF CO-AUTHORSHIP OF PUBLISHED WORK

I, Lisa Black, led the study design, data collection and analysis, and writing of this publication entitled 'Evaluation of enrofloxacin use in koalas (*Phascolarctos cinereus*) via population pharmacokinetics and Monte Carlo simulation'.

Merran Govendir oversaw the study and assisted with finalising the manuscript prior to publication. Cornelia Landersdorfer, Jürgen Bulitta and I co-developed and intensively discussed the pharmacokinetic model and performed the associated Monte Carlo simulations. Cornelia Landersdorfer and Jürgen Bulitta assisted with finalising the manuscript prior to publication. Joanna Griffith provided raw data generated following subcutaneous enrofloxacin administration for use in Monte Carlo simulations, and assisted with finalising the manuscript prior to publication.

Lisa Black



Date 31/7/2014

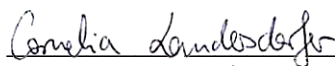
I, as co-author, endorse that this level of contribution by myself and the candidate indicated above is appropriate:

Merran Govendir



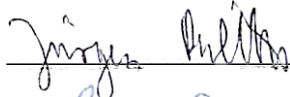
Date 31/7/2014

Cornelia Landersdorfer



Date 28/10/2013

Jürgen Bulitta



Date 28/10/2013

Joanna Griffith



Date 1/11/2013

CHAPTER 3. EVALUATION OF ENROFLOXACIN USE IN KOALAS (*PHASCOLARCTOS CINEREUS*) VIA POPULATION PHARMACOKINETICS AND MONTE CARLO SIMULATION

The following is a re-formatted manuscript, published by John Wiley and Sons Ltd:

Black, L.A., Landersdorfer, C.B., Bulitta, J.B., Griffith, J.E., Govendir, M. (2014) Evaluation of enrofloxacin use in koalas (*Phascolarctos cinereus*) via population pharmacokinetics and Monte Carlo simulation. Journal of Veterinary Pharmacology and Therapeutics, **37**, 301-311.

3.1 ABSTRACT

Clinically normal koalas (n = 6) received a single dose of intravenous enrofloxacin (10 mg/kg). Serial plasma samples were collected over 24 h and enrofloxacin concentrations were determined via HPLC. Population pharmacokinetic modelling was performed in S-ADAPT. The probability of target attainment (PTA) was predicted via Monte Carlo simulations (MCS) using relevant target values (30 to 300) based on the unbound area under the curve over 24 h divided by the MIC ($fAUC_{0-24}/MIC$), and published subcutaneous data were incorporated (Griffith et al., 2010). A two-compartment disposition model with allometrically scaled clearances (exponent: 0.75) and volumes of distribution (exponent: 1.0) adequately described the disposition of enrofloxacin. For 5.4 kg koalas (average weight), point estimates for total clearance

(SE%) were 2.58 L/h (15%), central volume of distribution 0.249 L (14%) and peripheral volume 2.77 L (20%). MCS using a target $fAUC_{0-24}/MIC$ of 40 predicted highest treatable MICs of 0.0625 µg/mL for intravenous dosing, and 0.0313 µg/mL for subcutaneous dosing of 10 mg/kg enrofloxacin every 24 h. Thus, the frequently used dosage of 10 mg/kg enrofloxacin every 24 h subcutaneously may be appropriate against gram-positive bacteria with MICs ≤ 0.03 µg/mL (PTA > 90%), but appears inadequate against gram-negative bacteria and *Chlamydiae* in koalas.

3.2 INTRODUCTION

The koala (*Phascolarctos cinereus*) is an iconic Australian marsupial, and the only surviving member of the Family Phascolarctidae (Lee & Carrick, 1989). In some Australian states the koala is listed as vulnerable, while isolated populations are endangered and many populations in New South Wales and Queensland are in decline (Natural Resource Management Ministerial Council, 2009; Department of Environment and Resource Management, 2012). Wild koalas frequently receive medical treatment in wildlife rehabilitation facilities across their natural range along the east coast of Australia, predominantly for wounds sustained in motor vehicle accidents, dog attacks, or due to disease (Department of Environment and Resource Management, 2011; Griffith et al., 2013).

Chlamydia pneumoniae, and the more highly pathogenic *Chlamydia pecorum* (Jackson et al., 1999), are the causative agents of chlamydiosis, which is the most common infectious disease in many koala populations (Stalder, 2003; Loader, 2010). Chlamydiosis can cause debilitating disease in koalas, resulting in one or more of the following: keratoconjunctivitis (Cockram & Jackson, 1976), a rhinitis/pneumonia

complex (Brown & Grice, 1984) and urogenital inflammation and fibrosis (Canfield, 1990; Obendorf & Handasyde, 1990) which can cause infertility. The veterinary registered fluoroquinolone, enrofloxacin, is used in some wildlife facilities to treat bacterial infections, including chlamydiosis (Blanshard & Bodley, 2008), and is anecdotally well tolerated by koalas at the commonly used dose of 10 mg/kg subcutaneously (s.c.) every 24 h (q 24 h). In other species, macrolides and tetracyclines are often used as first line anti-chlamydial drugs; however, these can induce a syndrome of inappetence, emaciation and death in koalas (Brown et al., 1984; Osawa & Carrick, 1990).

Enrofloxacin has a broad spectrum of aerobic antibacterial activity, including *Chlamydiae* (Walker & Dowling, 2006; Kumar et al., 2007), and displays concentration-dependent bacterial killing. Despite fluoroquinolones like ciprofloxacin having short half-lives of approximately 1.2 – 3.1 h in mice, they provide good activity in murine infection models when dosed every 24 h (Bédos et al., 1998). These properties favor fluoroquinolones like enrofloxacin for use in non-domesticated wildlife species where long dosing intervals are desirable, and where coverage is required for a variety of primary and secondary bacterial infections.

Little is known about the pharmacokinetics of enrofloxacin and its active metabolite, ciprofloxacin, in koalas, except that oral absorption is poor relative to subcutaneous absorption (Griffith et al., 2010). Additionally, multiple dosing of subcutaneous enrofloxacin at 10 mg/kg q 24 h to koalas with chlamydiosis resulted in a median unbound area under the plasma concentration-time curve from 0 to 24 h ($fAUC_{0-24}$) of 4.54 $\mu\text{g/mL}\cdot\text{h}$ (Griffith et al., 2010). With an assumed minimum inhibitory

concentration (MIC) of the target organisms of 0.25 µg/mL, this leads to a $fAUC_{0-24}/MIC$ ratio of 18.2, which is expected to be subtherapeutic against *Chlamydiae*. Of clinical relevance, this dose of 10 mg/kg s.c. q 24 h did not achieve microbiological cure when used to treat chlamydiosis, as shown by recurrence of chlamydial shedding after treatment withdrawal (Griffith, 2010), indicating a lack of efficacy. Due to the limited number of antibacterial agents that are tolerated by the koala, the ever-increasing problem of antibacterial resistance, and the lack of efficacy of enrofloxacin in treating koala chlamydiosis at the current dose of 10 mg/kg s.c. q 24 h (Griffith, 2010), it is prudent to use species specific pharmacokinetic data to optimise dosage regimens of antibacterials.

This study aimed to determine the population pharmacokinetics of enrofloxacin in clinically normal koalas after intravenous (i.v.) dosing. Additionally, the plasma concentrations of ciprofloxacin as the primary metabolite of enrofloxacin were measured. Our second objective was to determine the probability of achieving therapeutic targets following administration of a standard dose of 10 mg/kg enrofloxacin s.c. q 24 h to koalas, using Monte Carlo simulation (MCS) based on the developed population pharmacokinetic model and published minimal inhibitory concentrations (MICs) for *Chlamydiae*, and other bacteria likely to cause infections in koalas.

3.3 MATERIALS AND METHODS

3.3.1 Animals

Clinically normal koalas of both genders (n = 6; 2 female, 4 male), ranging in age from 15 months to nine years as determined by tooth wear (Martin, 1981) or previous

admission data, were recruited opportunistically from the Koala Hospital of the Koala Preservation Society of Australia (KPS), Port Macquarie, NSW and The Australia Zoo Wildlife Hospital (AZWH), Beerwah, Qld. Koalas had initially been hospitalised for illness or injury, presented as orphaned joeys and provided sanctuary while they matured, or had been rescued from dangerous situations (such as near major roads), and were being prepared for release back into the wild. Koalas were deemed healthy based on the results of a full physical examination conducted under general anaesthesia, and routine haematology and biochemistry analytes within reference ranges (Canfield et al., 1989; Blanshard, 1994). Koalas were housed in standard veterinary hospital enclosures and supplied with food *ad libitum* (various *Eucalyptus* spp.) and water throughout the study. This study was approved by The University of Sydney Animal Ethics Committee (protocol number N00/10-2007/4/4695).

General anaesthesia was induced by mask using 3 to 5% isoflurane in 100% oxygen to enable placement of a 20-gauge i.v. catheter into the cephalic vein for serial blood sampling. Enrofloxacin (Ilium Enrotril, Troy Laboratories, Smithfield, NSW), equivalent to 10 mg/kg, was diluted into a total volume of 5 mL using sterile water for injection and administered as an i.v. bolus over one minute. Serial blood samples (up to 1.5 mL) for determination of enrofloxacin concentration were collected into lithium heparin tubes (Vacurette®, Greiner Bio-One, Interpath Services Pty. Ltd, Victoria, Australia) at 0 (pre-dose), 2, 15, 30, 45 min, and 1, 1.5, 2, 4, 6, 8, 12 and 24 h. Blood was protected from light until centrifugation, which was performed within 1 h of collection. Plasma was transferred immediately into plain tubes and samples were stored at -20 °C and protected from light until analysis.

3.3.2 Analytical method

Plasma concentrations of enrofloxacin and ciprofloxacin were determined using a modified reversed-phase high-performance liquid chromatography (HPLC) method (Griffith et al., 2010), using an SPD-M20A photo-diode array detector (Shimadzu, Rydalmere, NSW, Australia) set at 275 nm. Data acquisition and peak integration were performed by Shimadzu Class VP software, version 7.4. Retention times for ciprofloxacin, enrofloxacin, and the internal standard (IS, difloxacin), were 7.87, 9.82 and 12.53 min, respectively. Pooled, drug-free koala plasma exhibited no interfering peaks.

A volume of 250 μL of each plasma sample was analysed in triplicate. Calibration standards (0.05, 0.5, 5, 50, 500 $\mu\text{g/mL}$ enrofloxacin; 0.01, 0.1, 1, 10, 100 $\mu\text{g/mL}$ ciprofloxacin) were prepared daily using pooled drug-free koala plasma to make serial dilutions from enrofloxacin and ciprofloxacin stock solutions. Analytical grade enrofloxacin, ciprofloxacin and difloxacin were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia) and dissolved in deionised water. Prior to solid phase extraction (SPE), 50 μL of a 50 $\mu\text{g/mL}$ solution of the IS was added to calibration standards and plasma samples.

Plasma samples underwent SPE as previously described (Griffith et al., 2010) and eluates were evaporated to dryness at 35 $^{\circ}\text{C}$ for 3 h using a vacuum concentrator (SPD 121P, Thermo Electron Corporation, Asheville, North Carolina, USA). The dried residue was reconstituted using 100 μL mobile phase, which consisted of acetonitrile (Lomb Scientific, Taren Point, NSW, Australia) and deionised water (50:50, v/v), 25 mM citric acid (Sigma-Aldrich, Castle Hill, NSW, Australia) and 10 mM sodium

dodecyl sulfate (Sigma-Aldrich, Castle Hill, NSW, Australia). Samples were centrifuged for 10 min at 14,000 g, and a volume of 10 μ L supernatant was injected into the HPLC system.

Recovery was $91.70 \pm 1.06\%$ for enrofloxacin and $80.50 \pm 3.89\%$ for ciprofloxacin, as determined by comparing three known concentrations spanning the calibration range of enrofloxacin or ciprofloxacin spiked into blank koala plasma prior to, and following, SPE. Interpolation of the daily calibration curve with a minimum coefficient of determination (R^2) > 0.999 was used to determine the concentration of enrofloxacin and ciprofloxacin in treated koala plasma. GenStat (14th edition; VSN International, 2011) was used to perform a lack-of-fit test, to evaluate linearity of the calibration curve for both drugs; the linear trend was significant for both curves (enrofloxacin: $P < 0.001$; ciprofloxacin: $P < 0.001$, determined via F-test).

The lower limit of detection (LLD) was 0.16 μ g/mL and the lower limit of quantification (LLQ) 0.48 μ g/mL for enrofloxacin. The LLD was 0.04 μ g/mL and the LLQ 0.13 μ g/mL for ciprofloxacin. These lower limits were determined using the standard deviation of the y-intercepts of the regression line (International Conference on Harmonisation, 2005). Intra-assay variation was assessed by the comparison of six replicates of five calibration standards spanning the assay range. Inter-assay variation was assessed by comparing replicates of three calibration standards spanning the range of the assay on each analysis day ($n = 4$). Intra- and inter-assay variation were $< 9.35\%$ and $< 10.2\%$ for enrofloxacin, and $< 10.6\%$ and $< 19.0\%$ for ciprofloxacin.

3.3.3 Non-compartmental analysis

Non-compartmental analysis was performed to estimate the bioavailability of enrofloxacin after s.c. dosing by incorporating previously published s.c. administration data (Griffith et al., 2010). Data were analysed using a standard non-compartmental pharmacokinetic approach, including only drug concentrations above the LLQ for enrofloxacin. The peak concentration ($C_{\max}$) was obtained directly from the measured concentrations and the terminal half-life ($t_{1/2}$) was determined by linear regression of the log concentration-time plots. The area under the concentration-time curve (AUC_{0-t}) was calculated to the last measurable concentration using the linear trapezoidal method. The terminal segment of the $AUC_{0-\infty}$ ($AUC_{\text{extrapolated}}$) was calculated as:

$AUC_{\text{extrapolated}} = C_{\text{last}} / k_{\text{el}}$, where C_{last} is the last measured plasma concentration, and k_{el} is the slope during the terminal phase.

The absolute bioavailability (F) after extravascular administration was calculated as:

$$F = (AUC_{\text{ev}} / AUC_{\text{iv}}) \times (\text{Dose}_{\text{iv}} / \text{Dose}_{\text{ev}}),$$

where AUC_{ev} is the median area under the curve estimated following extravascular administration (Griffith et al., 2010), and Dose_{ev} is the extravascular dose given to effect the AUC_{ev} .

3.3.4 Population pharmacokinetic analysis

Population pharmacokinetic modelling was performed to enable estimation of the between subject variability (BSV). BSV is important for performing MCS, which can simulate the probability of achieving therapeutic targets following antimicrobial administration. A non-linear mixed effects model was developed in S-ADAPT (version 1.57; Bauer, 2008) with the pre- and post-processing tool S-ADAPT-TRAN (Bulitta et al., 2011; Bulitta & Landersdorfer, 2011). The considered models described the one

minute infusion by a zero-order input into an infusion site compartment that was linked to the central compartment by a first-order mixing process. Models with one, two and three disposition compartments were considered. First order elimination and distribution were assumed. A log-normal distribution was used to describe BSV for all parameters. Models with and without a full variance-covariance matrix for clearance (CL) and volume parameters were evaluated. Body weight and plasma creatinine concentrations were considered as potential covariates that may affect the pharmacokinetics of enrofloxacin.

Visual predictive checks, residual plots and assessment of the S-ADAPT objective function were used to evaluate and compare models as described previously (Landersdorfer et al., 2012). For the visual predictive checks, enrofloxacin concentration-time profiles of 500 koalas were simulated for each of the competing models. The median, 50% prediction interval (25th to 75th percentiles) and 80% prediction interval (10th to 90th percentiles) were calculated from the simulated profiles and overlaid on the observed data. This allows an evaluation of whether the model adequately predicts the central tendency and variability in the observed concentrations.

3.3.5 Monte Carlo simulation

MCS was performed to simulate the disposition of enrofloxacin, taking BSV into account. These simulations were used to determine the proportion of koalas likely to achieve nominated $fAUC_{0-24}/MIC$ ratios of 30, 40, 75 and 300, based on the estimated population pharmacokinetic parameters. These $fAUC_{0-24}/MIC$ targets were chosen based on the following: fluoroquinolone $fAUC_{0-24}/MIC$ target values of 30 to 40 were associated with bacteriostasis at 24 h in *in vitro* models and murine infection models,

and microbiological cure in patients with infections by gram-positive bacteria (Lacy et al., 1999; Ambrose et al., 2001; Andes & Craig, 2002); an $fAUC_{0-24}/MIC$ of 75 was the target for microbiological cure in seriously ill patients using ciprofloxacin to treat infections caused by gram-negative pathogens (primarily *Pseudomonas aeruginosa*) (Forrest et al., 1993); and fluoroquinolone $fAUC_{0-24}/MIC$ ratios up to approximately 300 were required to suppress emergence of resistance against various pathogens (Tam et al., 2005; Tam et al., 2007).

A range of MICs from 0.03125 to 2 µg/mL were studied via MCS. The median s.c. bioavailability determined from the non-compartmental analysis (0.41) was used for assessment of s.c. doses. A protein binding value of 55.4% (Griffith et al., 2010) was used. Simulations of $fAUC_{0-24}$ were undertaken for 5000 subjects, using the estimated population mean and BSV for CL. For each MIC, the probability of target attainment (PTA) was calculated as the fraction of subjects who attained the respective pharmacokinetic/pharmacodynamic (PK/PD) target. The PK/PD breakpoint was defined as the highest MIC with a PTA of at least 90% (Frei et al., 2008; Landersdorfer et al., 2009). The PK/PD breakpoints were determined for $fAUC_{0-24}/MIC$ target values representing near-maximal bacterial killing and for resistance prevention of gram-positive and gram-negative bacteria (Forrest et al., 1993; Lacy et al., 1999; Ambrose et al., 2001; Tam et al., 2005; Tam et al., 2007).

3.4 RESULTS

Low ciprofloxacin concentrations (0.13 – 0.25 µg/mL) were found at 2 min in three of six koalas following i.v. enrofloxacin administration. Ciprofloxacin concentrations were below the LLQ at and after 15 min in these three koalas. There were detectable

ciprofloxacin concentrations throughout the study in the plasma of one koala, but these remained below the LLQ. The remaining koalas had no detectable ciprofloxacin in their plasma.

A two-compartment model with an additional compartment accounting for mixing between the infusion site and the central compartment described the present enrofloxacin data set well and was the best of the considered models. The area under the curve ($AUC_{0-\infty}$) predicted from the population pharmacokinetic parameter estimates (Table 3-1) for a 10 mg/kg i.v. dose was 20.9 $\mu\text{g/mL}\cdot\text{h}$ for a 5.4 kg koala (the average weight of koalas in the study), which matched the median $AUC_{0-\infty}$ from non-compartmental analysis closely (Table 3-2). The visual predictive check for the final model (Figure 3-1) indicated an adequate predictive performance of the population pharmacokinetic model; the median predicted concentration profile matched the central tendency of the observations reasonably well. Considering the small sample size of six koalas, the prediction intervals reasonably reflected the observed BSV (Figure 3-1), although the predicted variability was slightly larger than the observed variability at 4, 6 and 8 h post dose. Plasma creatinine concentrations were not found to be a predictive covariate of systemic CL. Gender neither affected body size adjusted CL, nor V_d . As our sample size was very small ($n = 6$), this covariate analysis was only for exploratory purposes. Allometric scaling by total body weight with a fixed exponent of 0.75 for CL and of 1.0 for the volume of distribution terms decreased the unexplained BSV by 11.7% for CL, 19.8% for V_c and 4.0% for V_p compared to a model without taking into account the effect of body weight.

Table 3-1. Population pharmacokinetic parameter estimates for enrofloxacin in koalas

Parameter (unit)	Parameter explanation	Population mean (%SE)	Between subject variability (%SE)
CL (L/h) ^a	Total body clearance	2.58 (15%) ^a	0.550 (46%)
V _c (L) ^a	Central volume of distribution	0.249 (14%)	0.774 (21%)
V _p (L) ^a	Peripheral volume of distribution	2.77 (20%)	1.22 (14%)
CL _d (L/h) ^a	Intercompartmental clearance	2.76 (28%)	1.69 (10%)
T _{1/2,mix} (min)	Half-life of mixing between the infusion site and the central compartment ^b	1.58 (21%)	0.579 (56%)
CV _{cp} (%)	Proportional residual error	1.41	
SD _{cp} (µg/mL)	Additive residual error	0.143	

^a: Clearances and volumes were scaled allometrically with an exponent of 0.75 for clearance and of 1.0 for volume of distribution terms based on a body weight of 5.4 kg (average weight of the koalas in the study).

^b: The bolus dose entered an injection site compartment that was linked to the central compartment with a first-order process. This model structure was necessary to adequately capture the concentration time-profiles in plasma after the bolus dose given over 1 min.

%SE: Relative standard error representing the precision of the estimated population mean and of the estimated between subject variability.

Table 3-2. Pharmacokinetic parameters [median (range)] from non-compartmental analysis after i.v. and s.c. administration of enrofloxacin.

	Enrofloxacin 10 mg/kg i.v. (n = 6)	Enrofloxacin 10 mg/kg s.c. (n = 10) (Griffith et al., 2010)
$C_{\max}$ (µg/mL)	72.9 (24.3 - 317)	2.08 (1.34 - 2.96)
$t_{1/2}$ (h)	1.79 (1.37 - 3.45)	2.31 (1.21 - 3.44)
$AUC_{0-4\text{ h}}$ (µg/mL.h)	15.8 (9.19 - 45.8)	4.82 (3.86 - 6.73)
$AUC_{0-\infty}$ (µg/mL.h)	20.3 (10.6 - 47.1)	8.32 (5.17 - 9.43)
$fAUC_{0-\infty}$ (µg/mL.h)	9.05 (4.73 - 21.0)	3.73 (2.31 - 4.21)
$F_{\text{s.c.}}$	-	0.41 (0.17 - 0.77)

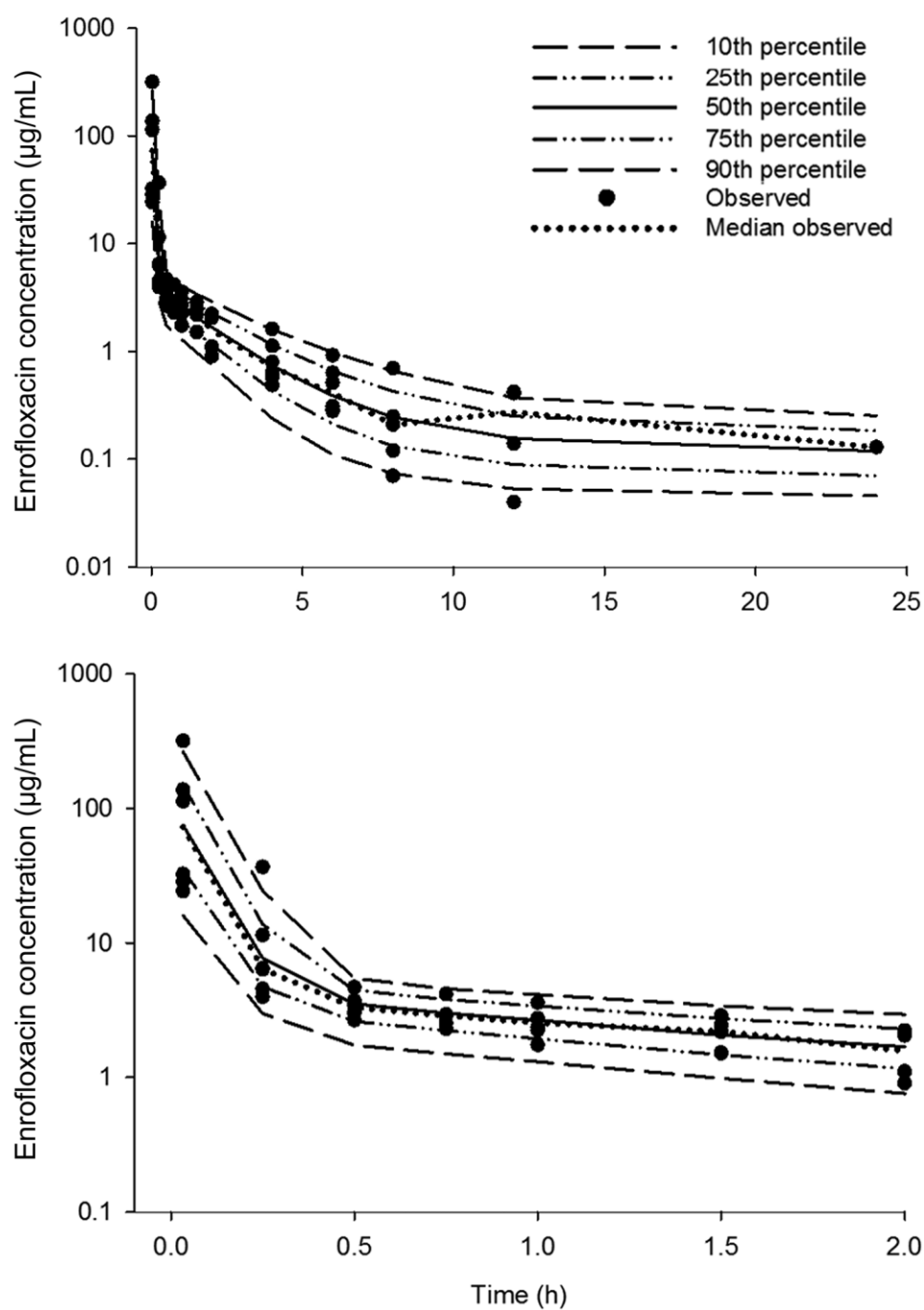


Figure 3-1. Visual predictive checks for enrofloxacin plasma concentrations over the whole observation period (upper panel) and the first 2 h (lower panel). The graphs show the individual observed concentrations, the median of the observed concentrations and the predicted percentiles from 500 simulated subjects. Ideally, 50% of the observed concentrations should be inside the interquartile range (i.e. between the 25th and 75th percentiles) at each time point, and 80% of the observed concentrations should be inside the 80% prediction interval (i.e. between the 10th and 90th percentiles).

MCS predicted low PTAs for MICs at or above 0.25 µg/mL for 10 mg/kg enrofloxacin i.v. or s.c. administered q 24 h (Figure 3-2). The PK/PD breakpoints for i.v. and s.c. dosing were low (Table 3-3), in particular for the target values $fAUC_{0-24}/MIC$ of 75 and 300.

Table 3-3. PK/PD breakpoints* for 10 mg/kg enrofloxacin i.v. or s.c. q 12 h and q 24 h.

$fAUC_{0-24}/MIC$ target	Intravenous administration q 24 h (µg/mL)	Subcutaneous administration q 24 h (µg/mL)	Intravenous administration q 12 h (µg/mL)	Subcutaneous administration q 12 h (µg/mL)
30	0.125	0.0625	0.25	0.125
40	0.0625	0.0313	0.125	0.0625
75	0.0313	0.0156	0.0625	0.0313
300	0.00781	0.00391	0.0156	0.00781

*The PK/PD breakpoint was defined as the highest MIC with a PTA of at least 90%.

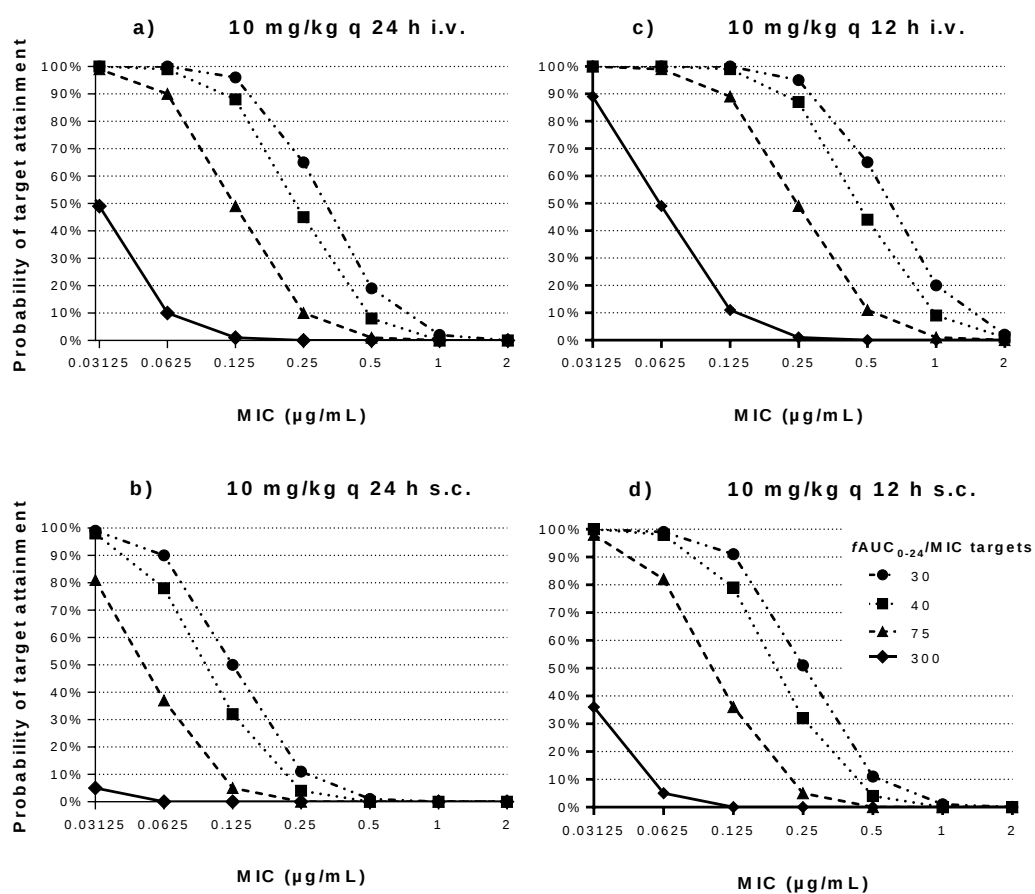


Figure 3-2. Probabilities of target attainment for selected $fAUC_{0-24}/MIC$ targets and MICs following administration of enrofloxacin 10 mg/kg a) intravenously q 24 h, b) subcutaneously q 24 h, c) intravenously q 12 h, and d) subcutaneously q 12 h.

For the proposed chlamydial target $fAUC_{0-24}/MIC$ of 75 and an MIC of 0.03125 µg/mL, the PTA was 80% after s.c. dosing and above 95% after i.v. dosing q 24 h. However, for an MIC of 0.25 µg/mL, the PTA was only 10% after i.v. dosing and 0% after s.c. dosing of 10 mg/kg enrofloxacin against chlamydial infections. When the dosing interval was decreased to q 12 h, the PTA was above 95% after i.v. dosing, and 81% after s.c. dosing for an MIC of 0.0625 µg/mL. Dosing 10 mg/kg q 12 h only increased the PTA to 50% for i.v. dosing and 5% for s.c. dosing for the proposed chlamydial MIC of 0.25 µg/mL.

There were no changes to demeanour, browsing habits or faecal output of any animals throughout the study, and there were no apparent adverse effects resulting from the general anaesthetic, i.v. enrofloxacin administration, i.v. catheter placement, frequent handling or hospitalisation.

3.5 DISCUSSION

This study provides further insight into the pharmacokinetics of enrofloxacin in koalas. The acquisition of i.v. data permitted estimation of previously unavailable pharmacokinetic parameters, such as total body CL and s.c. bioavailability. Combination of the i.v. data from this study with previously acquired s.c. pharmacokinetic data (Griffith et al., 2010), and incorporation of these data into a MCS, enabled assessment of whether the commonly used dose of 10 mg/kg s.c. q 24 h is likely to achieve PK/PD targets for aerobic bacterial infections, including chlamydiosis, in koalas.

A population pharmacokinetic approach was selected for this study as it enables analysis of data sets with high inter-individual variability, whilst allowing covariates

such as body weight and renal function to be quantitatively incorporated into the analysis. The main objective of the model was to predict PTAs for PK/PD targets using MCS. Thus, the model that best described the data in order to estimate CL, and subsequently AUC_{0-24} , was chosen as the superior model. This model benefitted from inclusion of an infusion site compartment to account for mixing (half-life: 1.58 min) between the infusion site and the central compartment. This may have been caused by the rapid 1-min infusion. The developed population pharmacokinetic model for enrofloxacin in the koala provided precise curve fits (Figure 3-3) and yielded a good predictive performance (Figure 3-1). All parameters were estimated with adequate precision (relative standard errors below 30% for all population typical values). Even after accounting for the effect of body size on CL and volume of distribution via an allometric body size model, there was considerable random BSV with a coefficient of variation of 55.0% for total CL and 77.4% for volume of the central compartment (Table 3-1). This highlights the need for using MCS methodology to account for BSV in pharmacokinetics for optimising dosage regimens.

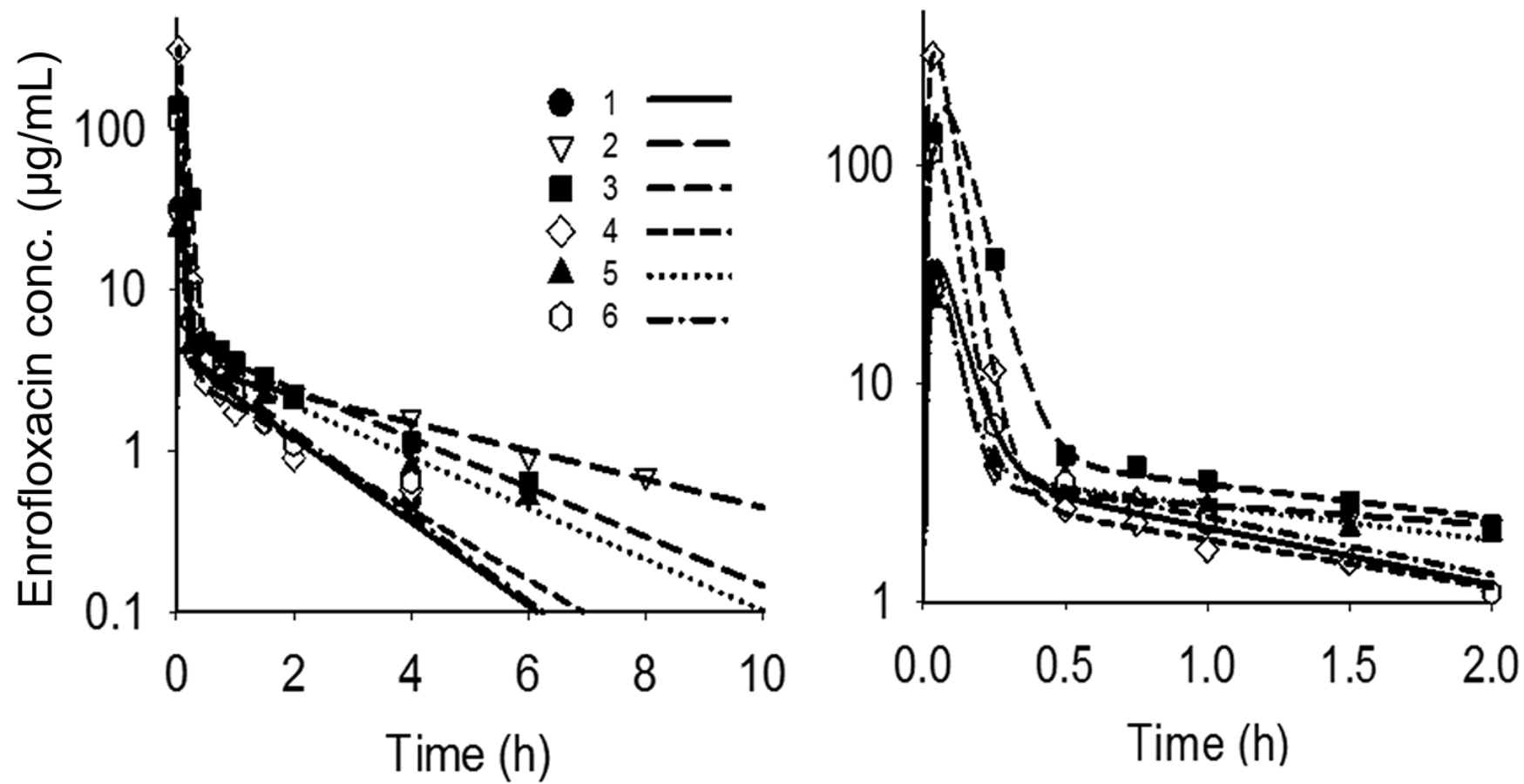


Figure 3-3. Individual fitted and observed plasma concentrations of enrofloxacin for each koala for the first 10 h (a) and the first 2 h (b). Only concentrations above the LLQ are shown.

The CL estimated for koalas was within the range of estimates reported for many species, and was noticeably lower than in some species (Nielsen & Gyrd-Hansen, 1997; Knoll et al., 1999; Bermingham & Papich, 2002; Rao et al., 2002; Gandolf et al., 2005; Peyrou et al., 2006; Elmas et al., 2007; Idowu et al., 2010) (Table 3-4). This is an important finding in a species, such as the koala, that is able to rapidly metabolise and eliminate some chemical compounds (Pass & Brown, 1990; Kimble et al., 2013). This enhanced metabolism is thought to be an adaptation to enable detoxification of the koala's *Eucalyptus* spp. diet, which is high in toxic plant secondary metabolites (Cork & Foley, 1997) and has the potential to render some drugs unsuitable for use in this species.

Table 3-4. Comparison of median (range), mean $\pm$ SD, or mean $\pm$ SE§ values for CL in selected species. * mean values. # population pharmacokinetic values. For this table, population pharmacokinetic values were scaled linearly to allow interspecies comparisons.

Species	CL (L/h/kg)
Koala (present study)	0.48 [#]
Alpaca (Gandolf et al., 2005)	0.08 (0.04 – 0.12)
Beef steer (Idowu et al., 2010)	0.70 $\pm$ 0.02
Chicken (Knoll et al., 1999)	0.62*
Dairy cow (Idowu et al., 2010)	1.45 $\pm$ 0.12
Goat (Rao et al., 2002)	1.33 $\pm$ 0.94 [§]
Horse (Peyrou et al., 2006)	0.38 $\pm$ 0.09
Pig (Nielsen & Gyrd-Hansen, 1997)	0.37 $\pm$ 0.11
Rabbit (Elmas et al., 2007)	1.7 $\pm$ 0.52
Sheep (Bermingham & Papich, 2002)	0.20 (0.13 – 0.22)

Prior to renal elimination, many species metabolise enrofloxacin to an active metabolite, ciprofloxacin (Kaartinen et al., 1995; Mengozzi et al., 1996; Cester & Toutain, 1997; Kaartinen et al., 1997; Seguin et al., 2004). As ciprofloxacin and enrofloxacin both

contribute activity against susceptible bacteria *in vitro* (Lautzenhiser et al., 2001), the concentration of this active metabolite should be considered when assessing enrofloxacin efficacy. Ciprofloxacin peaks were only detected in the chromatograms of four koalas (67%) and these were only quantifiable at the first sampling time (2 min). Approximately 20 to 35% of administered enrofloxacin is converted to ciprofloxacin in horses (Karttinen et al., 1997), 40% in dogs (Cester & Toutain, 1997) and 60% in cattle (Idowu et al., 2010). Low plasma concentrations of ciprofloxacin have been detected in pigs (Nielsen & Gyrd-Hansen, 1997), foals (Bermingham et al., 2000) and chickens (Knoll et al., 1999) following systemic enrofloxacin administration. The peak concentrations of ciprofloxacin were approximately 500-fold lower than those of enrofloxacin in koalas, implying metabolism from enrofloxacin to ciprofloxacin is less extensive in koalas than in other species. This may indicate a different metabolic pathway for enrofloxacin, or rapid metabolism or excretion of ciprofloxacin (Easmon & Crane, 1985a; Easmon & Crane, 1985b; Hawkins et al., 1998; Cole et al., 2009). Due to the low plasma concentrations of ciprofloxacin detected in koalas, the antimicrobial activity of this active metabolite was considered negligible, thus ciprofloxacin was not included in the pharmacodynamic assessment of enrofloxacin.

The primary PK/PD index associated with fluoroquinolone efficacy is the $fAUC_{0-24}/MIC$ ratio (Walker & Dowling, 2006), which varies between pathogens. In the current study, target $fAUC_{0-24}/MIC$ ratios of 30, 40, 75 and 300 were investigated, based on approximate unbound fractions of published fluoroquinolone targets for gram-positive [~ 30 to 40, (Lacy et al., 1999; Ambrose et al., 2001)], and gram-negative [~ 75 (Forrest et al., 1993)] organisms, as well as the target associated with prevention of antibacterial resistance [300 (Tam et al., 2005; Tam et al., 2007)]. Although *Chlamydiae* are atypical

bacteria, they are more closely related to gram-negative than gram-positive bacteria (Caldwell et al., 1981), so the gram-negative target of 75 might be more reflective of *Chlamydiae* and was considered here. However, the targets of 30 or 40 may be relevant for less seriously ill koalas with an intact immune system.

The *in vitro* susceptibility of koala isolates of *C. pecorum* and *C. pneumoniae* to enrofloxacin have not been determined. However, the MIC and minimum bactericidal concentration (MBC) of enrofloxacin against *C. pneumoniae* isolated from another Australian marsupial, the western barred bandicoot, were both 0.25 to 0.5 µg/mL (Kumar et al., 2007), and the enrofloxacin MIC against *Chlamydia psittaci* isolated from turkeys was 0.25 µg/mL (Butaye et al., 1997). As the *in vitro* susceptibility of *C. pecorum* to antibacterials is similar across chlamydial species (Kumamoto et al., 1992; Pudjiatmoko et al., 1998), 0.25 µg/mL was selected as the target MIC for anti-chlamydial dosing calculations in this study.

Due to the frequency of dog bite wounds seen in koala hospital admissions (Griffith et al., 2013), MIC values for aerobic bacteria known to be part of the normal canine oral flora and commonly isolated from dog bite wounds (Bailie et al., 1978) were also considered. These included *Pasteurella multocida*, *Staphylococcus epidermidis*, *Streptococcus* spp., *Bacillus* spp. and Enterobacteriaceae. MIC values for canine and feline isolates of some of these pathogens range from 0.03 to 0.25 µg/mL (Popova & Todorov, 2008; Schink et al., 2013). Additionally, the enrofloxacin susceptibility breakpoint for common feline and canine dermal, respiratory and urinary pathogens, including canine Enterobacteriaceae and *Staphylococcus* spp., is ≤ 0.5 µg/mL (Clinical

and Laboratory Standards Institute, 2008), hence the required enrofloxacin dose for treating chlamydiosis is likely to be similar to that required for some of these pathogens.

MCS was performed to determine the PTA of $fAUC_{0-24}/MIC$ ratios for the above-mentioned PK/PD targets and MICs. Our MCS did not account for BSV in oral bioavailability, since we only had a non-compartmental estimate for the average bioavailability. The PK/PD breakpoints for enrofloxacin would have been even lower than the already low breakpoints reported in Table 3-3, if BSV for bioavailability was included. For gram-positive pathogens with a low MIC of 0.0313 $\mu\text{g/mL}$, 10 mg/kg enrofloxacin s.c. q 24 h appears to be an appropriate dose, with PTAs of 99% and 98% at $fAUC_{0-24}/MIC$ targets of 30 and 40. However, 10 mg/kg enrofloxacin s.c. q 24 h cannot be recommended for gram-positive infections with MICs of 0.125 $\mu\text{g/mL}$ and higher, gram-negative infections with MICs of 0.0313 $\mu\text{g/mL}$ and higher, or for preventing emergence of bacterial resistance during therapy, as these situations had PTAs below 90%. Increasing the dosage to 10 mg/kg q 12 h had only a limited effect on these PTAs, with the PTA for gram-positive infections with MICs of 0.125 $\mu\text{g/mL}$ increasing to 91%, and the highest treatable MIC for gram-negative infections increasing to 0.0313 $\mu\text{g/mL}$. It should be noted that administering 10 mg/kg q 12 h compared to 10 mg/kg q 24 h may increase the risk of host toxicity due to increased drug exposure, and such dose increases should be approached with caution.

The PK/PD breakpoints for i.v. administration of 10 mg/kg enrofloxacin q 24 h indicate that gram-negative organisms with low MICs of $\leq 0.0313 \mu\text{g/mL}$, and gram-positive organisms with slightly higher MICs of 0.0625 to 0.125 $\mu\text{g/mL}$, may be successfully treated. When administered q 12 h, 10 mg/kg enrofloxacin i.v. appears suitable for

gram-negative infections with MICs ≤ 0.0625 $\mu\text{g/mL}$, and for gram-positive infections with MICs up to 0.125 to 0.25 $\mu\text{g/mL}$. However, i.v. dosing greatly increases the risk of toxicity due to the attainment of substantially higher C_{max} and on average 2.4-fold higher $\text{AUC}_{0-\infty}$ values (Table 3-2) compared with s.c. dosing. Thus, administering enrofloxacin q 12 h i.v. cannot be advocated until safety trials have been performed, and may carry a high risk with limited pharmacodynamic benefits.

For the assumed chlamydial MIC of 0.25 $\mu\text{g/mL}$, the PTA of the proposed s.c. $f\text{AUC}_{0-24}/\text{MIC}$ target of 75 was 0% for 10 mg/kg enrofloxacin q 24 h. When administered i.v., the PTA for *Chlamydiae* was 10%, which is well below the breakpoint of 90%; increasing the dosage to 10 mg/kg q 12 h still resulted in PTAs substantially below the breakpoint. Clearly, doses well in excess of 10 mg/kg q 24 h would be required to reach a $\text{PTA} \geq 90\%$ for *Chlamydiae*. Such doses were not calculated in this study due to concerns of toxicity. Fluoroquinolones like enrofloxacin have extensive tissue penetration due to their physicochemical properties and are potentially toxic at high doses. The koala is a monogastric hindgut fermentor with the largest relative caecal surface area of any species (Snipes et al., 1993). Hindgut fermentors are particularly susceptible to changes in the gastrointestinal microflora induced by antimicrobial therapy (Toutain et al., 2010), which is thought to be the reason for the inappetence and wasting observed following administration of macrolides and tetracyclines to koalas (Brown et al., 1984; Osawa et al., 1993). Thus, in the absence of clinical data demonstrating safety of high doses of enrofloxacin in koalas, increasing the dose sufficiently to enable attainment of these pharmacodynamic targets appears inappropriate.

Although chlamydial load decreased markedly during treatment with enrofloxacin in clinical and subclinical koalas treated at 10 mg/kg s.c. q 24 h, chlamydial load increased after treatment withdrawal, despite apparent resolution of clinical signs (Griffith, 2010). Recurrence of chlamydial shedding following antibiotic therapy is concerning, and highlights treatment failure, which is supported by this study. Another concern is the potential emergence of resistance when subtherapeutic doses are used. This can occur rapidly and frequently with fluoroquinolone use (Fink et al., 1994; Papich & Riviere, 2009b), and is problematic due to the current value of enrofloxacin as a broad spectrum antibiotic in veterinary medicine. These factors, in conjunction with the low PTAs determined in this study, indicate that reassessment of enrofloxacin as a valid treatment option for koalas with chlamydiosis, or other bacterial infections that are likely to have MICs above the breakpoints outlined in Table 3-3, is required. Importantly, an alternative antibiotic that is not routinely used systemically for other veterinary species or humans, chloramphenicol, appears to clear *Chlamydiae* in koalas following treatment (Markey et al., 2007; Govendir et al., 2012), and should be considered for treatment of this disease. As for enrofloxacin, the potential toxicity associated with chloramphenicol use needs to be considered.

Although stable antibiotic resistance has not been identified in human chlamydial infections (Sandoz & Rockey, 2010), fluoroquinolone resistance can develop *in vitro* when *Chlamydiae* are continuously exposed to subinhibitory concentrations (Morrissey et al., 2002). Hence, treatment of koalas with enrofloxacin could potentially lead to the development of resistant *Chlamydiae*, as well as other resistant strains of bacteria from sites such as the integument or gastrointestinal system. This is of particular concern for a species, such as the koala, in which antibiotic treatment options are already limited.

This study provides the first population pharmacokinetic analysis for enrofloxacin in koalas. The associated MCS yielded low PK/PD breakpoints and supported previous evidence that 10 mg/kg enrofloxacin s.c. q 24 h is inadequate for treating koalas with chlamydiosis (Griffith, 2010). Further studies will be required to determine the *in vitro* MICs for enrofloxacin against koala isolates of *C. pecorum* and *C. pneumoniae*. When such information becomes available for these or other enrofloxacin-susceptible bacteria infecting koalas, the data presented in the present MCS plots will enable an informed decision as to the appropriate use of enrofloxacin for these infections.

CONFIRMATION OF CO-AUTHORSHIP OF PUBLISHED WORK

I, Lisa Black, led the study design, data collection and analysis, and writing of this publication entitled 'Pharmacokinetics of fluconazole following intravenous and oral administration to koalas (*Phascolarctos cinereus*)'.

Merran Govendir oversaw the study and assisted with finalising the manuscript prior to publication. Mark Krockenberger provided expertise regarding cryptococcosis, and provided assistance with finalising the manuscript prior to publication. Benjamin Kimble provided assistance with the initial HPLC assay development and validation, and assistance with finalising the manuscript prior to publication.

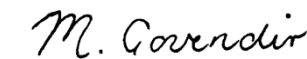
Lisa Black



Date 31/7/2014

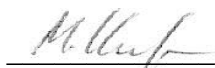
I, as co-author, endorse that this level of contribution by myself and the candidate indicated above is appropriate:

Merran Govendir



Date 31/7/2014

Mark Krockenberger



Date 31/7/2014

Benjamin Kimble



Date 31/7/2014

CHAPTER 4. PHARMACOKINETICS OF FLUCONAZOLE

FOLLOWING INTRAVENOUS AND ORAL

ADMINISTRATION TO KOALAS (*PHASCOLARCTOS*

CINEREUS)

The following is a re-formatted manuscript, published by John Wiley and Sons Ltd:

Black, L.A., Krockenberger, M.B., Kimble, B., Govendir, M. (2013) Pharmacokinetics of fluconazole following intravenous and oral administration to koalas (*Phascolarctos cinereus*). Journal of Veterinary Pharmacology and Therapeutics, **37**, 90-98.

4.1 ABSTRACT

Clinically normal koalas (n = 12) received a single dose of 10 mg/kg fluconazole orally (p.o.; n = 6) or intravenously (i.v.; n = 6). Serial plasma samples were collected over 24 h and fluconazole concentrations were determined using a validated HPLC assay. A non-compartmental pharmacokinetic analysis was performed. Following i.v. administration, median (range) plasma clearance (CL) and steady state volume of distribution (V_{ss}) were 0.31 (0.11 – 0.55) L/h/kg and 0.92 (0.38 – 1.40) L/kg, respectively. The elimination half-life ($t_{1/2}$) was much shorter than in many species (i.v.: median 2.25, range 0.98 – 6.51 h; p.o.: 4.69, range 2.47 – 8.01 h) and oral bioavailability was low and variable (median 0.53, range 0.20 – 0.97). Absorption rate-limited disposition was evident. Plasma protein binding was $39.5 \pm 3.5\%$. Although fluconazole volume of distribution (V_{area}) displayed an allometric relationship with other mammals, CL and $t_{1/2}$ did not. Allometrically scaled values were approximately seven-fold lower (CL) and six-fold higher ($t_{1/2}$) than observed values, highlighting flaws

associated with this technique in physiologically distinct species. On the basis of $fAUC/MIC$ pharmacodynamic targets, fluconazole is predicted to be ineffective against *Cryptococcus gattii* in the koala as a sole therapeutic agent administered at 10 mg/kg p.o. every 12 h.

4.2 INTRODUCTION

Cryptococcosis is a worldwide, life-threatening, systemic fungal disease of both humans and animals, and is the second most prevalent infectious disease of koalas (Stalder, 2003). Cryptococcosis in koalas is caused by *Cryptococcus gattii* and, unlike disease induced by *C. neoformans* in humans, is not thought to be associated with overt host immunocompromise (Krockenberger et al., 2005). Asymptomatic carriage of cryptococcal organisms in the nasal mucosa of koalas occurs frequently (Connolly et al., 1999; Krockenberger et al., 2002b) and has been attributed to the close affiliation between koalas and *Eucalyptus* spp. trees, which are natural hosts for *C. gattii* (Ellis & Pfeiffer, 1990; Pfeiffer & Ellis, 1992). The interaction between host, pathogen and environment is complex, and occasionally results in clinical disease, affecting approximately 2.5% of koalas necropsied at The University of Sydney between 1980 and 2003 (Stalder, 2003). Disease usually affects the respiratory tract, although dissemination is common, with CNS involvement detected in 37% of cases in one study (Krockenberger et al., 2003).

Fluconazole is an antifungal drug currently administered orally to treat cryptococcosis in koalas (Wynne et al., 2012), although pharmacokinetic parameters have not been established in this species. Previous studies in koalas have demonstrated low plasma concentrations of both the fluoroquinolone antibacterial, enrofloxacin (Griffith et al.,

2010), and the non-steroidal anti-inflammatory drug, meloxicam (Kimble et al., 2013) following oral administration of conventional doses. Therefore, to properly establish the utility of fluconazole in the management of cryptococcosis in koalas it is imperative to characterise fluconazole pharmacokinetics, including oral bioavailability.

The current published dose rate for fluconazole in koalas for nasopharyngeal cryptococcosis is 50 - 100 mg orally (p.o.) twice daily (b.i.d.) (Blanshard & Bodley, 2008), which equates to approximately 5 - 10 mg/kg. Doses in excess of 15 - 20 mg/kg p.o. b.i.d. are sometimes advised by one of the authors (MK), and doses as high as 25 mg/kg p.o. b.i.d. have been reported (Wynne et al., 2012). Intravenous (i.v.) dosing at 15 mg/kg once daily (s.i.d.) has also been used (A. Gillett, pers. comm.)². The aim of this study was to characterise the pharmacokinetics of fluconazole in clinically normal koalas after oral and i.v. dosing, and to assess the suitability of the oral dosing regimen of 10 mg/kg b.i.d. This dose was chosen as it approximates both the suggested dose rate for koalas (Blanshard & Bodley, 2008), and the canine/feline doses upon which koala drug doses are traditionally based.

4.3 MATERIALS AND METHODS

4.3.1 Animals

Twelve clinically normal koalas of both genders (8 males, 4 females; average weight 5.0 ± 2.0 kg), ranging in age from 15 months to 10 years as determined by tooth wear (Martin, 1981) or from management records, were recruited for the study. Koalas were sourced from Billabong Koala and Wildlife Park (BKWP), Port Macquarie, NSW and

² Personal communication: Amber Gillett, Wildlife Veterinarian, Australia Zoo Wildlife Hospital, Qld, May 2013.

the Australia Zoo Wildlife Hospital (AZWH), Beerwah, Qld. Koalas from BKWP were from a display collection; those sourced from AZWH were wild koalas that had previously been hospitalised for illness or injury, or had presented as orphaned joeys and were being provided sanctuary while they matured. Koalas from AZWH were being prepared for release back into the wild. Koalas were deemed healthy based on a full physical examination performed under general anaesthesia by veterinarians at AZWH, or by recent clinical observations (captive koalas at BKWP) and haematology and biochemistry analytes within reference ranges (Canfield et al., 1989; Blanshard, 1994). All koalas were free of cryptococcal disease, as determined by a latex cryptococcal agglutination test (LCAT; Crypto-LA[®], Wampole Laboratories, Cranbury, NJ, USA) and absence of clinical signs of disease. Koalas were housed in standard veterinary hospital enclosures (AZWH) or standard display enclosures (BKWP) and supplied with *ad libitum* food (various *Eucalyptus* spp.) and water for the duration of the study. The study was approved by The University of Sydney Animal Ethics Committee (protocol number N00/10-2007/4/4695).

A 20-gauge i.v. catheter was placed into the cephalic vein for serial blood sampling. Catheters were placed under manual restraint at BKWP, or under general anaesthesia induced by mask using 3 – 5% isoflurane in 100% oxygen at AZWH. Anaesthetised koalas were recovered for one to two hours before drug administration commenced at $t = 0$.

Koalas were randomly divided into p.o. ($n = 6$) and i.v. ($n = 6$) treatment groups. The i.v. group received 10 mg/kg fluconazole (Fluconazole Hexal[®], Sandoz Pty Ltd, Pyrmont, NSW, Australia) administered as a 30 min i.v. infusion, as per the

manufacturer's instructions, using a syringe pump. The p.o. group received 10 mg/kg fluconazole (Bova Compounding Chemist, Caringbah, NSW, Australia) mixed with Infasoy paste (Wyeth Australia, Baulkham Hills, NSW, Australia) immediately prior to being orally administered using a syringe. Serial blood samples (up to 1.2 mL) for fluconazole plasma concentration determination were collected into lithium heparin tubes at the following time points: $t = 0, 0.5, 1, 2, 4, 6, 8, 12$ and 24 h for the p.o. study, and $t = 0, 0.5, 1, 1.5, 2.5, 4.5, 6.5, 8.5, 12.5$ and 24.5 h for the i.v. study. Samples were centrifuged within 1 h of collection and plasma was transferred into plain tubes. Plasma was stored at -20°C and protected from light until analysis.

4.3.2 Analytical method

Plasma concentrations of fluconazole were determined using a modified reversed-phase high performance liquid chromatography (HPLC) assay (Zhang et al., 2008). The HPLC system (Shimadzu, Rydalmere, NSW, Australia) comprised a Shimadzu LC-20AT solvent delivery unit, SIL-20AC autosampler, DGU-20A5 degassing unit, CTO-20AC column oven maintained at 25°C , and an SPD-M20A diode array detector. The chromatographic separation was achieved using an Apollo C-18, $5\text{ }\mu\text{m}$ ($250\text{ mm} \times 4.6\text{ mm ID}$) column (Grace Davison Discovery Sciences, Rowville, Victoria, Australia) in conjunction with an Opti-guard 1 mm C-18 pre-column (Optimize Technologies, Oregon City, OR, USA). The isocratic mobile phase consisted of acetonitrile (Lomb Scientific, Taren Point, NSW, Australia) and deionised water (30:70, v/v), and was delivered at a rate of 1.0 mL/min . Target analyte absorption was monitored at 210 nm . Data acquisition and peak integration were performed using Shimadzu Class VP software, version 7.4. Retention times for fluconazole and the internal standard (IS),

phenacetin, were 5.14 and 11.63 min respectively. Pooled, drug-free koala plasma was free of interfering peaks at these retention times.

Triplicate 100 μ L plasma samples from treated koalas were analysed. Calibration standards (0.033, 0.067, 0.33, 0.67, 3.33, 6.67, 33.3, 66.7 μ g/mL) were prepared daily using pooled drug-free koala plasma to prepare serial dilutions from a 1,000 μ g/mL fluconazole stock solution (analytical grade fluconazole, Sigma-Aldrich, Castle Hill, NSW, Australia) dissolved in analytical grade methanol (Lomb Scientific, Taren Point, NSW, Australia). Prior to solid phase extraction (SPE), 50 μ L of 25 μ g/mL phenacetin (Sigma-Aldrich, Castle Hill, NSW, Australia) and 800 μ L of 100 mM phosphate buffer (pH 6.0) were added to calibration standards and plasma samples.

Plasma samples and calibration standards underwent SPE using an Oasis HLB 1 cm³ cartridge (Waters, Milford, MA, USA) via a vacuum manifold. SPE columns were conditioned with 1 mL methanol followed by 1 mL 100 mM phosphate buffer. Plasma supernatant (900 μ L) was loaded, and columns were washed with 1 mL 100 mM phosphate buffer followed by 1 mL 85% phosphate buffer (100 mM phosphate buffer in methanol, 85:15, v/v). Samples were then eluted with 1.5 mL methanol. Eluates were evaporated to dryness at 35 °C for 3.5 h using a vacuum concentrator (SPD 121P, Thermo Electron Corporation, Asheville, NC, USA), and reconstituted with 100 μ L mobile phase. Samples were centrifuged for 10 min at 14,000 x g, and 15 μ L supernatant was injected into the HPLC system in duplicate.

Fluconazole recovery was $90 \pm 0.03\%$, determined by comparing replicates of three known fluconazole concentrations spanning the calibration range spiked into blank koala plasma prior to, and following, SPE.

Interpolation of the daily calibration curve with a minimum coefficient of determination (R^2) ≥ 0.999 was used to determine the concentration of fluconazole in treated koala plasma. A lack-of-fit test was also performed using GenStat 14th edition (VSN International, 2011) to verify linearity of the calibration curve, and showed no deviation from the linear model ($P < 0.001$, determined via an F-test). The lower limit of detection (LLD) and lower limit of quantification (LLQ) were calculated as 0.03 $\mu\text{g/mL}$ and 0.10 $\mu\text{g/mL}$ respectively, using the standard deviation of the y-intercepts of the regression line (International Conference on Harmonisation, 2005). Intra-assay variation was assessed by comparing six replicates of five calibration standards spanning the assay range on a single day. Inter-assay variation was assessed by comparing replicates of three calibration standards spanning the assay range on three analysis days. Intra- and inter-assay variation, expressed as % RSD, were $< 10.25\%$ and $< 13.37\%$, respectively.

4.3.3 Plasma protein binding

The fraction of fluconazole bound to plasma proteins was determined by ultrafiltration (Dow, 2006). The plasma pH and protein concentration of freshly collected, pooled drug-free plasma from six koalas were determined prior to the assay. Analytical grade fluconazole was added to 1 mL plasma aliquots to yield concentrations of 0.67, 6.67 and 66.67 $\mu\text{g/mL}$. Samples were incubated at 36 °C to replicate koala core body temperature (Degabriele & Dawson, 1979) for 30 min, and centrifuged in a fixed angle rotor centrifuge (Eppendorf 5417C; Eppendorf, Hamburg, Germany) at 4,000 x g for 30 min,

using an ultrafiltration device (Amicon® Ultra-0.5 30 kDa, Merck Millipore, Kilsyth, Victoria, Australia). The non-protein bound ultrafiltrate ($\text{Drug}_{\text{unbound}}$) was recovered and the fluconazole concentration determined. This was compared with the initial concentration added prior to ultrafiltration ($\text{Drug}_{\text{total}}$). Samples were prepared and analysed in triplicate. Percentage binding to plasma proteins was determined using the following equation:

$$\% \text{ protein binding} = \left(\frac{\text{Drug}_{\text{total}} - \text{Drug}_{\text{unbound}}}{\text{Drug}_{\text{total}}} \right) \times 100$$

4.3.4 Pharmacokinetic analysis

A standard non-compartmental pharmacokinetic approach was used for data analysis, using only values above the LLQ. The maximal concentration (C_{max}) and time to reach maximal concentration (t_{max}) were determined from the raw data. The terminal rate constant (k_{el}) was determined as the gradient of the terminal portion of the natural log of the plasma concentration-time curve. Following oral administration, k_{el} was assumed to be the absorption constant (k_{a}) due to absorption rate-limited disposition with this route of administration (Yáñez et al., 2011). The area under the concentration-time curve (AUC_{0-t}) and area under the first moment curve (AUMC_{0-t}) were calculated to the last quantifiable concentration using the linear trapezoidal method. The elimination half-life ($t_{1/2}$), systemic clearance (CL), volume of distribution at steady state (V_{ss}), volume of distribution during pseudoequilibrium (V_{area}), mean residence time (MRT), bioavailability (F), terminal segments of the $\text{AUC}_{0-\infty}$ and $\text{AUMC}_{0-\infty}$ ($\text{AUC}_{\text{extrapolated}}$ and $\text{AUMC}_{\text{extrapolated}}$ respectively), and unbound $\text{AUC}_{0-\infty}$ ($f\text{AUC}_{0-\infty}$) were calculated using the following equations:

$$t_{1/2} = \ln 2 / k_{\text{el}}$$

$$CL = \text{dose} / AUC_{0-\infty}$$

$$V_{ss} = CL \times MRT$$

$$V_{area} = CL / k_{el}$$

$$MRT = AUMC_{0-\infty} / AUC_{0-\infty}$$

$F = (AUC_{ev} / AUC_{iv}) \times (Dose_{iv} / Dose_{ev})$, where AUC_{ev} is the median AUC_{0-t} calculated following extravascular administration, and $Dose_{ev}$ is the extravascular dose given to effect the AUC_{ev} .

$AUC_{extrapolated} = C_{last} / k_{el}$, where C_{last} is the last measured plasma concentration.

$AUMC_{extrapolated} = [(C_{last} \times t_{last}) / k_{el}] + (C_{last} / k_{el}^2)$, where C_{last} is the last measured plasma concentration, and t_{last} is the time of the last measured plasma concentration.

$$fAUC_{0-\infty} = AUC_{0-\infty} \times (1 - \frac{\% \text{ protein binding}}{100})$$

Previously derived allometric coefficients and exponents for fluconazole $t_{1/2}$, CL and V_d (Jezequel, 1994) were applied to the body weight of the koalas using the following equation:

$y = a \cdot (BW)^b$, where y is the pharmacokinetic parameter being scaled ($t_{1/2}$, CL or V_d); BW is the median body weight (kg) of koalas used in this study; and a and b are the allometric coefficients and exponents previously determined for fluconazole (Jezequel, 1994). V_d was assumed to be V_{area} , as it was derived from $t_{1/2}$ and CL data for each species (Jezequel, 1994). Theoretical values determined allometrically were compared with observed values for koalas, as well as observed values for other species, to determine the feasibility of allometric scaling of fluconazole in koalas.

4.3.5 Antifungal susceptibility testing

Fluconazole MICs for 10 koala *C. gattii* isolates were determined *in vitro*. These isolates had been cultured from koalas between 1992 and 2012 and stored at -80 °C or freeze-dried. Isolates were subcultured twice onto Sabouraud dextrose agar prior to susceptibility testing. Susceptibility testing was performed by IMVS Pathology, South Australia, using the Sensititre® YeastOne® microbroth dilution test panel.

4.3.6 Statistical analysis

A Spearman's rank correlation coefficient was calculated to determine whether pharmacokinetic values correlated with age, body weight or plasma creatinine concentrations. A Mann-Whitney test was used for comparison of pharmacokinetic parameters between treatment groups. For all tests, statistical significance was accepted at $P < 0.05$.

4.4 RESULTS

The pharmacokinetic parameters estimated for fluconazole are summarised in Table 4-1. The semi-logarithmic plasma concentration-time curves following p.o. and i.v. administration to healthy koalas are presented in Figure 4-1. In conjunction with the disparity in terminal $t_{1/2}$ estimates between the different routes of administration, these curves are consistent with absorption-rate limited disposition of orally administered fluconazole ('flip-flop' kinetics). The i.v. plasma drug concentration-time curve showed a biphasic decline, with distribution equilibrium reached within 30 min of cessation of the infusion ($t = 1$ h). The $AUC_{\text{extrapolated}}$ across both treatment groups was $< 16.87\%$ (median 4.61; range 0.72 - 16.87) of the $AUC_{0-\infty}$. The final quantifiable fluconazole concentrations in plasma were detected at $t = 4.5$ h ($n = 2$), $t = 6.5$ h ($n = 1$), $t = 12.5$ h

(n = 2) and t = 24.5 h (n = 1) for the i.v. group, and t = 8 h (n = 1), t = 12 h (n = 3) and t = 24 h (n = 2) for the p.o. group. Fluconazole plasma protein binding was $39.5 \pm 3.5\%$, and was linear over the range 0.67 – 66.67 µg/mL.

Table 4-1. Pharmacokinetic parameters (median and range) estimated following administration of fluconazole i.v. and p.o. to clinically normal koalas. * k_a was calculated as the terminal rate constant, due to absorption rate-limited disposition.

	Fluconazole	Fluconazole
	10 mg/kg i.v. (n = 6)	10 mg/kg p.o. (n = 6)
C_{max} (µg/mL)	20.63 (10.15 - 35.96)	2.00 (1.21 – 4.81)
t_{max} (h)	-	7.00 (4.00 – 8.00)
$t_{1/2}$ (h)	2.25 (0.98 – 6.51)	4.69 (2.47 – 8.01)
k_{el} (h ⁻¹)	0.40 (0.11 – 0.71)	-
k_a (h ⁻¹)	-	0.15 (0.09 – 0.28)*
CL (L/h/kg)	0.31 (0.11 – 0.55)	-
V_{ss} (L/kg)	0.92 (0.38 – 1.40)	-
V_{area} (L/kg)	1.17 (0.60 – 1.60)	-
$AUC_{0-\infty}$ (µg/mL.h)	34.54 (18.26 – 88.08)	22.87 (8.48 – 41.83)
$fAUC_{0-\infty}$ (µg/mL.h)	20.90 (11.05 – 53.31)	13.84 (5.13 – 25.32)
$AUMC_{0-\infty}$ (µg/mL.h ²)	112.33 (19.15 – 987.82)	250.17 (68.49 – 830.11)
MRT (h)	3.05 (0.97 – 11.21)	11.10 (8.07 – 19.84)
F	-	0.53 (0.20 – 0.97)

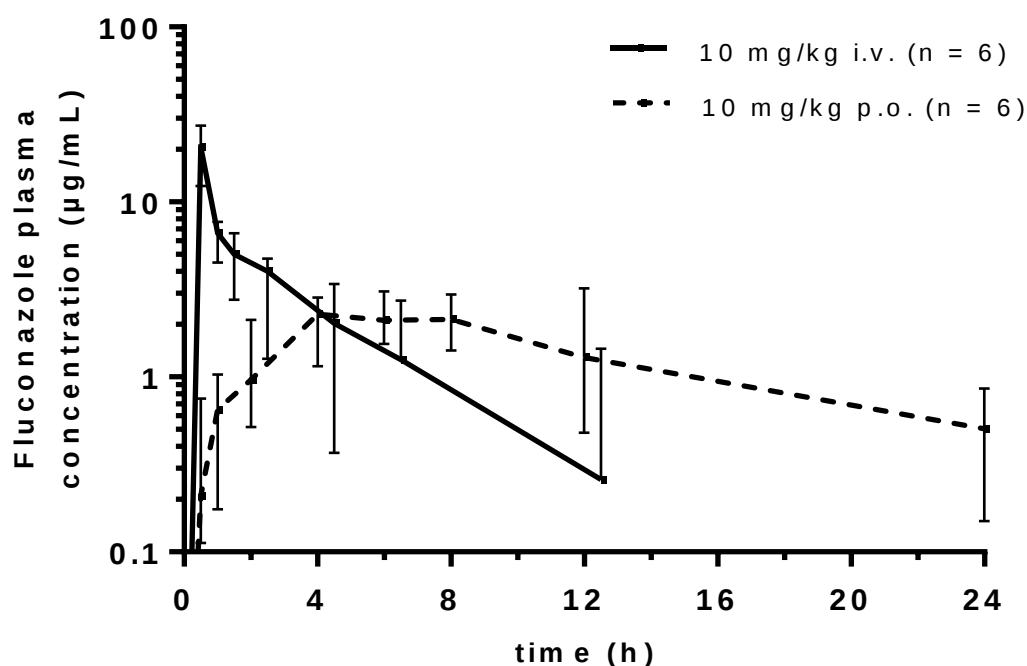


Figure 4-1. Semilogarithmic plasma-concentration time curves (median $\pm$ IQR) for fluconazole following i.v. and p.o. administration. LLQ = 0.1 $\mu\text{g/mL}$.

Oral administration resulted in a significantly lower C_{max} and a longer MRT ($P = 0.005$ and $P = 0.043$, respectively) than i.v. administration. No statistically significant differences were detected between the other pharmacokinetic parameters common to both p.o. and i.v. treatment groups. Values for k_{el} , $t_{1/2}$ and $\text{AUMC}_{0-\infty}$ showed a correlation with body weight ($\rho = -0.83$, 0.83 and 0.83 respectively) in the i.v. treatment group, although this was not statistically significant ($P = 0.05$ for all). No other parameters in either treatment group displayed a significant correlation with body weight, age, or plasma creatinine concentration. The theoretical V_{area} derived using allometric scaling was similar to the median observed value (Table 4-2). CL and $t_{1/2}$ did not show an allometric relationship with the seven species used for comparison (Figure 4-2), with allometrically scaled values being approximately six-fold higher ($t_{1/2}$) and seven-fold lower (CL) than observed values (Table 4-2). There were no changes to the demeanour, browsing habits or faecal output of any koalas during or following the

study, and there were no apparent adverse effects resulting from drug administration, i.v. catheter placement, frequent handling or hospitalisation.

Table 4-2. Comparison of observed and allometrically scaled pharmacokinetic parameters for koalas using previously determined coefficients and exponents for fluconazole (Jezequel, 1994). Estimates based on the median weight of koalas in this study, 4.48 kg.

Parameter	Observed value	Allometrically scaled value
V_{area} (L)	5.25	3.21
CL (L/h)	1.37	0.20
$t_{1/2}$ (h)	2.25	12.47

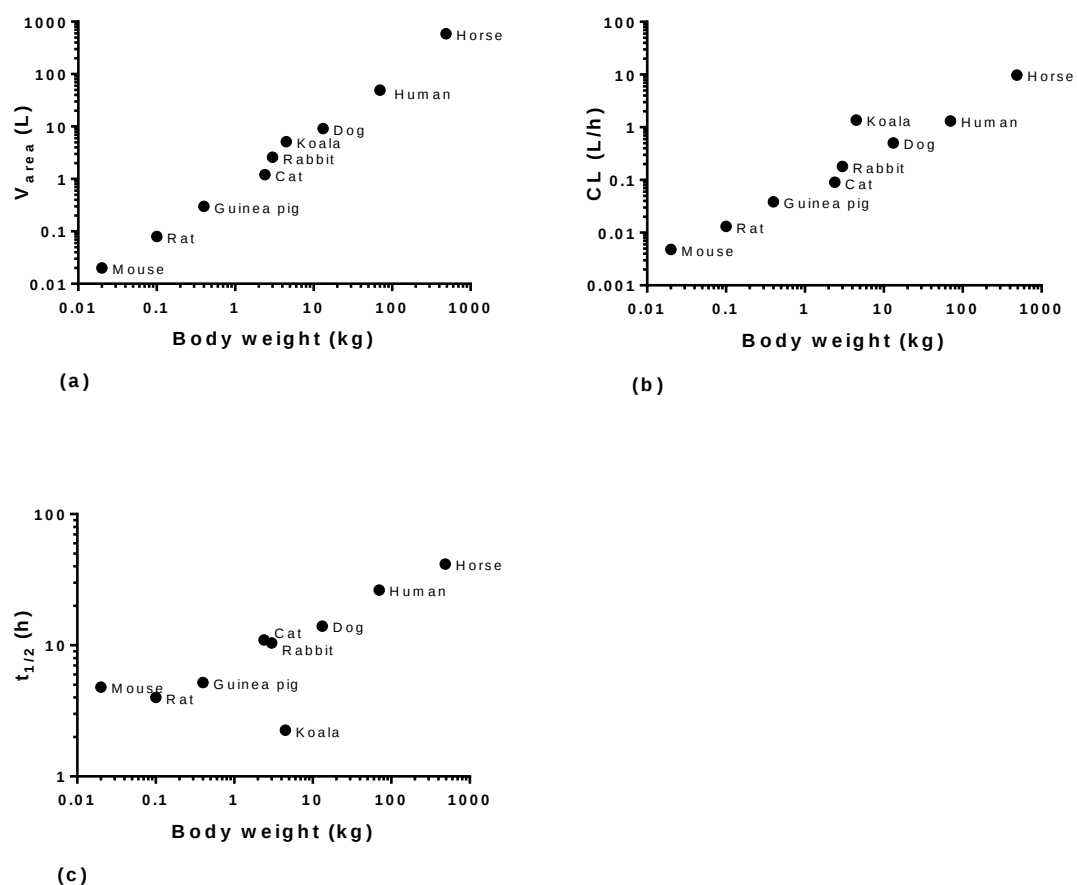


Figure 4-2. Allometric plots of log fluconazole V_{area} (a), CL (b) and $t_{1/2}$ (c) versus log body weight in nine species. Data points for the mouse, rat, guinea pig, rabbit, cat, dog and human taken from a previous allometric study (Jezequel, 1994). Data points for the horse taken from Latimer et al (2001).

In vitro fluconazole MIC values for *C. gattii* isolates varied from 1 to 16 $\mu\text{g/mL}$. Both the MIC_{50} and MIC_{90} were 4 $\mu\text{g/mL}$. The median (range) $f\text{AUC}/\text{MIC}$ ratios calculated for twice daily dosing at 10 mg/kg were 6.92 (2.57 – 12.66) for oral administration, and 10.45 (5.52 – 26.64) for i.v. administration.

4.5 DISCUSSION

In many species, the low molecular weight, long terminal $t_{1/2}$, large volume of distribution (V_d), almost complete oral bioavailability and low degree of protein binding

make fluconazole a sound choice for treating cryptococcosis, allowing good penetration into the cerebrospinal fluid (CSF) with the convenience of once daily oral dosing. However, this study demonstrated marked differences in $t_{1/2}$, CL, F and plasma protein binding for koalas compared with other mammals. This makes it difficult to predict pharmacokinetic parameters for koalas based on allometric scaling, which is an important finding for a drug that tends to show an allometric relationship across species (Jezequel, 1994). There was also clear evidence for absorption rate-limited disposition of orally administered fluconazole, which has not been described in other species. The data from this study enabled an assessment of the magnitude of the $AUC_{0-\infty}$ following administration of 10 mg/kg fluconazole p.o. and i.v., the parameter that is most closely linked to therapeutic outcomes of fluconazole therapy (Klepser et al., 1998).

There was very little difference between estimated values for V_{ss} and V_{area} , indicating only a small fraction of fluconazole is eliminated prior to reaching pseudoequilibrium (Toutain & Bousquet-Mélou, 2004b). Values for V_{ss} only marginally exceeded total body water volume (Nagy & Martin, 1985; Ellis & Carrick, 1992), implying tissue concentrations are likely to approximate plasma concentrations. An allometric relationship for fluconazole V_{area} was observed between the koala and other species (mice, rats, guinea pigs, cats, rabbits, dogs, humans and horses) (Jezequel, 1994; Latimer et al., 2001); this may be attributed to fluconazole being distributed throughout body water in all species studied, and is consistent with fluconazole V_{area} being considered an invariant parameter across species (Jezequel, 1994). However, an allometric relationship did not exist between koalas and other species for CL and $t_{1/2}$. Fluconazole is eliminated principally by the kidney, and the prolonged $t_{1/2}$ observed in many species has been attributed to net tubular reabsorption (Davis et al., 2009). The

close allometric relationships of CL and $t_{1/2}$ amongst the above-mentioned species, excluding the koala, imply that the extent of tubular reabsorption is fairly consistent across these species. Thus, a decreased extent of net tubular reabsorption may be responsible for the more rapid CL and shorter $t_{1/2}$ of fluconazole observed in koalas. There are other possible explanations for the anomalous elimination rate in koalas, such as active tubular secretion and biotransformation. These may have evolved as adaptive mechanisms to enable this folivorous marsupial to detoxify its eucalypt diet (Cork & Foley, 1997). It should be noted that the presence of an interspecies allometric relationship with a high R^2 value does not mean that interspecies dose extrapolation based on allometry is an appropriate method of dose determination. There can be large errors associated with interspecies allometric scaling, even in the presence of a high degree of correlation (Mahmood & Balian, 1996; Martinez et al., 2006b; Hunter & Isaza, 2008). However, allometry has been introduced here to emphasise the degree of error that may be encountered when interspecies dose extrapolation is used in exotic species without careful consideration of its limitations and assumptions, which have been reviewed elsewhere and can be easily overlooked (Hunter & Isaza, 2008).

Oral bioavailability of fluconazole amongst koalas was variable, and lower than the almost complete bioavailability reported for mice, dogs, cats, horses and humans (Humphrey et al., 1985; Brammer et al., 1990; Craig et al., 1994; Latimer et al., 2001). The t_{max} in this study was recorded later than the 1 - 4 h recorded for most species (Humphrey et al., 1985; Ripa et al., 1993; Craig et al., 1994; Latimer et al., 2001; Davis et al., 2009). These observations reflect both a limited rate and extent of oral fluconazole absorption in koalas. Similar findings have been reported in koalas for another orally administered antimicrobial, enrofloxacin (Griffith et al., 2010), and very

low bioavailability of orally administered meloxicam has been reported in koalas (Kimble et al., 2013). Possible explanations for the limited extent and rate of oral xenobiotic absorption in koalas include extensive first-pass metabolism of oral xenobiotics due to enhanced liver function (Pass & Brown, 1990; Liapis et al., 2000; Ngo et al., 2000) or metabolism by enzymes within the gastrointestinal wall (George, 1981; Ilett et al., 1990); binding of xenobiotics by gastrointestinal contents such as cellulose, as has been described in horses and sheep (Lees et al., 1988; van Duijkeren et al., 1996); or an inability of oral drugs to reach the gastrointestinal wall, due to the densely packed, finely masticated material that fills the proximal gastrointestinal tract (Blanshard & Bodley, 2008). These are all speculative, and further research is required to investigate the reason for the limited oral absorption that is becoming apparent in this species.

The *in vitro* fluconazole MIC for *C. gattii* is generally much higher than that of many antifungals, including amphotericin B and many azoles (Klepser et al., 1998; Burgess & Hastings, 2000; Trilles et al., 2004; Torres-Rodríguez et al., 2008; Mendes et al., 2010; Espinel-Ingroff et al., 2012). However, it shows good *in vivo* activity, presumably due to its low degree of protein binding, long $t_{1/2}$, low molecular weight and large V_d . Additionally, the *in vivo* efficacy of fluconazole against CNS mycoses has been attributed to its pharmacokinetic properties, namely the high water solubility and low protein binding (10 - 12% in most species) (Humphrey et al., 1985) that allow penetration into the CSF and most bodily fluids (Foulds et al., 1988; Walsh et al., 1989; Vaden et al., 1997; Latimer et al., 2001). Fluconazole protein binding in koalas is approximately four times greater than in other documented species (Humphrey et al., 1985), and may negatively impact antifungal activity when used to treat CNS

cryptococcosis by impeding fluconazole's ability to cross the blood-brain barrier (Bailey et al., 1990). Further assessment of CSF penetration and clinical response data are required to assess these speculations.

To our knowledge, pharmacodynamic targets for fluconazole against *C. gattii* have not been derived, so targets determined for *C. neoformans* were used. Fluconazole displays concentration-independent fungistasis, and the pharmacodynamic index associated with antifungal activity for *C. neoformans* is the AUC/MIC ratio (Klepser et al., 1998). The $AUC_{0-\infty}$ was used for AUC/MIC estimations, as this is equivalent to the AUC at steady state during a single dosing interval (Pillai & Venkataramanan, 2012) and this was doubled to account for twice daily dosing. The MIC_{90} of 4 $\mu\text{g/mL}$ and the unbound fraction ($fAUC_{0-\infty}$) were used. The $fAUC/MIC$ ratio (median 6.92; range 2.57 – 12.66) achieved in this study following administration of fluconazole 10 mg/kg p.o. falls markedly short of the *C. neoformans* $fAUC/MIC$ target of 192 (Burgess & Hastings, 2000), which is equivalent to maintaining an average plasma concentration of 8 $\mu\text{g/mL}$ throughout the dosing interval (Toutain et al., 2007). Another *in vitro* study found 0.5 x MIC as the concentration of maximal *C. neoformans* suppression (Klepser et al., 1998); maintaining an average plasma concentration of 0.5 x MIC corresponds to a much lower $fAUC/MIC$ ratio of 12. This target was achieved in only one of six koalas in this study following p.o. administration, and in three of six koalas following i.v. administration. A $fAUC/MIC$ ratio of 12 sits above the 95th percentile of the p.o. $fAUC/MIC$ distribution in this study, indicating fewer than 5% of koalas are likely to reach this target. However, this increases to 45% with i.v. administration. As fluconazole displays linear pharmacokinetics (Louie et al., 1998a; Louie et al., 1998b), it is unlikely that the dose of 25 mg/kg p.o. reported previously (Wynne et al., 2012) would reach the $fAUC/MIC$

target of 192 in any koalas, although the lower target of 12 may be attainable in some koalas. It should be noted that these targets are not definitive and can only be used as a guide to therapy, as antifungal susceptibility results are known to vary widely between laboratories and strains of *C. gattii* (Rex et al., 1993; Rex et al., 2001; Gomez-Lopez et al., 2008).

In treating koalas with cryptococcosis, fluconazole is often administered concurrently with amphotericin B in an attempt to improve treatment outcomes. Amphotericin B is associated with nephrotoxicity, and reduces renal blood flow and glomerular filtration rate of most subjects during treatment (Burgess & Birchall, 1972; Tolins & Raij, 1988). If fluconazole is eliminated renally in koalas, concurrent amphotericin B administration may result in a slower elimination rate and longer elimination $t_{1/2}$ than in healthy koalas. Thus, a pharmacokinetic study should be undertaken in clinical animals receiving both fluconazole and amphotericin B to detect any changes in the fluconazole pharmacokinetic profile under treatment conditions.

This study demonstrates important differences in fluconazole pharmacokinetic parameters between koalas and many previously studied species, including a shorter $t_{1/2}$, lower bioavailability, a higher degree of protein-binding and absorption rate-limited disposition. Importantly, koalas are poorly represented by the allometric relationship that has been proposed for fluconazole amongst other species, which highlights the errors that can be made when interspecies allometric scaling is employed without careful consideration of the technique's limitations and required assumptions (Hunter & Isaza, 2008). The commonly used dose of 10 mg/kg p.o. b.i.d. failed to reach pharmacodynamic targets in this study, and it appears that attaining the target

*f*AUC/MIC of 192 using fluconazole as a sole therapeutic agent will be difficult. Due to the limited oral bioavailability, i.v. dosing may be advantageous although higher doses need to be investigated, and the logistics of long-term i.v dosing would need to be considered. In light of the failure to reach pharmacodynamic targets at the dose of 10 mg/kg p.o. as a sole therapeutic agent, and the clinical bias to use concurrent amphotericin B in clinical disease in order to improve therapeutic outcomes, the investigation of fluconazole pharmacodynamics when administered in conjunction with amphotericin B needs to be undertaken.

CONFIRMATION OF CO-AUTHORSHIP OF WORK

SUBMITTED FOR PUBLICATION

I, Lisa Black, led the study design, data collection and analysis, and writing of this manuscript entitled ‘*In vitro* activities of chloramphenicol, florfenicol and enrofloxacin against *Chlamydia pecorum* isolated from koalas (*Phascolarctos cinereus*).’

Merran Govendir oversaw the study and assisted with finalising the manuscript prior to submission. Damien Higgins offered advice regarding the study design and technical aspects of cell culture, as well as assistance finalising the manuscript prior to submission.

Lisa Black



Date 31/7/2014

I, as co-author, endorse that this level of contribution by myself and the candidate indicated above is appropriate:

Merran Govendir



Date 31/7/2014

Damien Higgins



Date 31/7/2014

CHAPTER 5. *IN VITRO* ACTIVITIES OF CHLORAMPHENICOL, FLORFENICOL AND ENROFLOXACIN AGAINST *CHLAMYDIA PECORUM* ISOLATED FROM KOALAS (*PHASCOLARCTOS* *CINEREUS*)

The following is a re-formatted manuscript, currently being reviewed for publication.

5.1 ABSTRACT

Chlamydiosis is a debilitating disease of koalas in Australia and no information exists regarding the antimicrobial susceptibility of *Chlamydia pecorum* or *C. pneumoniae* isolated from koalas. The *in vitro* susceptibility of koala isolates of *Chlamydia pecorum* to enrofloxacin, chloramphenicol and florfenicol was determined by culturing three stored isolates and seven clinical swabs. Susceptibility testing was undertaken using cycloheximide-treated Buffalo Green Monkey Kidney cells in 96 well microtitre plates. The MICs of enrofloxacin, chloramphenicol and florfenicol for all isolates ranged from 0.25 – 0.50 µg/mL, 1 - 2 µg/mL, and 1 - 2 µg/mL, respectively. MBC values for five isolates were also determined, and were within one two-fold dilution of MICs. The MICs and MBCs of these antimicrobials were within ranges previously reported for other chlamydial species. When combined with previously published pharmacokinetic data, the *in vitro* susceptibility results support chloramphenicol as a more appropriate treatment option than enrofloxacin for koalas with chlamydiosis. The susceptibility

results also indicate florfenicol may be an appropriate treatment option for koalas with chlamydiosis, and warrants further investigation.

5.2 INTRODUCTION

Chlamydiosis is a common and debilitating disease of koalas (*Phascolarctos cinereus*), with infection rates of up to 85% detected in some populations (Jackson et al., 1999). The aetiological agents are *Chlamydia pecorum* and *C. pneumoniae* (Glassick et al., 1996). *C. pecorum* is the more pathogenic of the two, being implicated in the majority of cases of significant disease (Jackson et al., 1999). By contrast, *C. pneumoniae* tends to be implicated in less severe disease, and sole *C. pneumoniae* infections are often low-grade or asymptomatic (Jackson et al., 1999; Griffith, 2010). Clinical disease induced by *C. pecorum* and *C. pneumoniae* in koalas consists of a spectrum of ocular (Cockram and Jackson, 1974), urogenital (McColl et al., 1984) and respiratory disease (Brown and Grice, 1984). Whilst the latter has been incompletely described, ocular disease can result in severe keratoconjunctivitis (Cockram and Jackson, 1974) and urogenital disease can lead to debilitating cystitis, infertility, and ascending pyelonephritis (Hemsley and Canfield, 1997; Higgins et al., 2005).

Koalas with chlamydiosis are presented frequently to wildlife hospitals for treatment (Griffith et al., 2013). Two antimicrobials are used commonly for systemic treatment: chloramphenicol and enrofloxacin. These antimicrobials have been chosen as, at conventional dosages (Blanshard and Bodley, 2008), they do not induce the syndrome of inappetence, emaciation, and death that has been reported in koalas following administration of the first-line anti-chlamydial drugs, the macrolides and tetracyclines (Brown et al., 1984; Osawa and Carrick, 1990). Chloramphenicol and enrofloxacin

dosages for koalas have been developed by extrapolation from dogs and cats, or by trial and error. Recently, pharmacokinetic studies of enrofloxacin and chloramphenicol in koalas have been conducted (Griffith et al., 2010; Black et al., 2012; Govendir et al., 2012; Black et al., 2013;) as a starting point for koala-specific dosage development for chlamydiosis. However, a common limitation to these studies was the lack of available data regarding the *in vitro* susceptibility of koala *C. pecorum* isolates to chloramphenicol or enrofloxacin. Although *in vitro* antimicrobial susceptibilities are not always reflective of the susceptibility *in vivo*, such data are important in the development of dosage regimens, as they are used as a basis for establishing target antimicrobial plasma concentrations that are theoretically therapeutic.

Although ruminant *C. pecorum* isolates have undergone susceptibility testing to a range of antimicrobials (Pudjiatmoko et al., 1998), the susceptibility of *C. pecorum* to enrofloxacin and chloramphenicol has not been tested. This paucity of information is likely due to the expense and technical difficulties associated with antimicrobial susceptibility testing of intracellular pathogens, and the lack of standardised guidelines for performing antimicrobial susceptibility testing against *Chlamydia* spp. In order to better establish theoretical therapeutic targets for koalas with chlamydiosis, this study aimed to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of enrofloxacin, chloramphenicol, and the chloramphenicol derivative, florfenicol, against koala *C. pecorum* isolates. Florfenicol was selected as a potential alternative to chloramphenicol, due to the recent market withdrawal of the commercial veterinary chloramphenicol formulation in Australia.

5.3 MATERIALS AND METHODS

5.3.1 *Chlamydia pecorum* isolates

Ten *C. pecorum* isolates from koalas were tested. The koala *C. pecorum* type strain (MC/Mars Bar) (Marsh et al., 2011) was kindly donated by the Institute of Health and Biomedical Innovation, Queensland University of Technology. Two archived *C. pecorum* isolates that had been cultured from koalas at Featherdale Wildlife Park in Western Sydney, NSW, in 1990 were used. Seven isolates were grown from ocular and urogenital swabs (aluminium shafted rayon swabs; Copan, Brescia, Italy) collected from wild koalas presenting with ocular or urogenital signs of chlamydiosis to the Currumbin Sanctuary Wildlife Hospital, Currumbin, Queensland between November 2012 and February 2013. Swabs were stored at -80 °C in 2-SP (0.2 M sucrose, 0.02 M phosphate) supplemented with 10% foetal bovine serum (FBS), 50 µg/mL gentamicin, 100 µg/mL vancomycin, and 25 µg/mL amphotericin B until cultured. A plain swab was also taken from each site to confirm presence of *C. pecorum* and absence of *C. pneumoniae* by real-time PCR (qPCR).

5.3.2 *Chlamydia pecorum* species identification

DNA extraction was undertaken using the Bioline Isolate II blood and genomic DNA kits (Bioline, Alexandria, NSW, Australia) according to the manufacturer's instructions. *C. pecorum* and *C. pneumoniae* were detected using an existing species-specific qPCR method (Govendir et al., 2012), revalidated for use with SsoAdvanced™ SYBR® Green Supermix (Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, Australia) and run using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, Australia).

5.3.3 Cell culture and growth of Chlamydial isolates from swabs

Isolates were grown in Buffalo Green Monkey Kidney (BGMK) cells, which were maintained in Dulbecco's Modified Eagle's Medium (Hyclone DMEM; Thermo-Fisher Scientific, Scoresby, Victoria, Australia) containing 4.5 g/L glucose. Immediately prior to use, the medium was supplemented with 9% FBS (In Vitro Technologies, Noble Park North, Victoria, Australia), 20 mM HEPES buffer (In Vitro Technologies, Noble Park North, Victoria, Australia), and 2 mM L-glutamine (Sigma-Aldrich, Castle Hill, NSW, Australia). Flasks were maintained at 37 °C in 5% CO₂. For the initial inoculation of BGMK cells with *Chlamydia* spp. from clinical swabs, 40 µg/mL gentamicin, 150 µg/mL vancomycin, and 5 µg/mL amphotericin B were added to the medium. Isolates were then passaged four times in antimicrobial-free medium prior to susceptibility testing.

5.3.4 *In vitro* susceptibility testing

Chloramphenicol, enrofloxacin, florfenicol and ofloxacin (all from Sigma-Aldrich, Castle Hill, NSW, Australia) were obtained as powders and solubilised, after accounting for purity.

Twenty-four hours prior to inoculation, each well of a 96-well microtitre plate was seeded with 100 µL medium containing 3.0×10^5 BGMK cells/mL. The next day, wells were inoculated with 100 µL of the test isolate diluted to yield 10^3 to 10^4 inclusion-forming units per mL. Plates were centrifuged at $2000 \times g$ for 1 h, and then incubated at 37 °C in 5% CO₂ for 1 h. Following centrifugation, wells were aspirated and overlaid with 200 µL medium containing 1 µg/mL cycloheximide (Sigma-Aldrich, Castle Hill, NSW, Australia) and serial two-fold dilutions of the test antimicrobial. For MIC

determination, plates were incubated for 48 h at 37 °C in 5% CO₂, prior to fixing and staining with a fluorescein-conjugated genus-specific *Chlamydia*-lipopolysaccharide monoclonal antibody (Cellabs, Brookvale, NSW, Australia) according to the manufacturer's instructions. The MIC was defined as one two-fold dilution above the transition-point MIC (MIC_{TP}), which was defined as the concentration of drug resulting in altered size or morphology of at least 90% of inclusions (Suchland et al., 2003). For MBC determination, plates were incubated in 5% CO₂ for 48 h at 37 °C after addition of the antimicrobial. The medium was then aspirated and cells were washed twice with phosphate buffered saline. Antibiotic free medium containing 1 µg/mL cycloheximide was added and plates were frozen at -80 °C, thawed, passed onto new monolayers as described above and incubated for 48 h at 37 °C in 5% CO₂. Monolayers were then fixed and stained as above. The MBC was defined as the lowest concentration of antimicrobial that prevented inclusion formation after a single freeze-thaw passage. All tests were run in triplicate. *C. trachomatis* (ATCC VR-879, serotype H) was donated by the Westmead Institute for Clinical Pathology and Medical Research (ICPMR) and used as the reference strain.

5.3.5 *Mycoplasma* testing of cultures

Each *C. pecorum* isolate was tested for *Mycoplasma* spp. contamination at the first passage, and again immediately prior to use in susceptibility testing, using a commercial *Mycoplasma* PCR detection kit (Applied Biological Materials Inc., Richmond, BC, Canada) according to the manufacturer's instructions. The maintenance flasks of BGMK cells were also tested periodically, including immediately prior to and upon completion of susceptibility testing.

5.4 RESULTS

The seven clinical swabs collected from koalas presenting to the Currumbin Sanctuary Wildlife Hospital were positive for *C. pecorum*, but negative for *C. pneumoniae*. All isolates were negative for *Mycoplasma* spp. on each testing occasion. The BGMK cell maintenance flask was consistently *Mycoplasma* spp. negative on PCR, and remained so at the conclusion of susceptibility testing.

For each antimicrobial, the MIC values were within one two-fold dilution across all ten isolates. For the five isolates used in MBC determination, results were within one two-fold dilution of each other (Table 5-1). For each isolate that underwent MBC testing, the MBC was within one two-fold dilution of the MIC. Susceptibility results were consistent between isolates from different locations (South East Queensland and Western Sydney), and between isolates collected 23 years apart (1990 and 2013).

Table 5-1. *In vitro* activity of chloramphenicol, enrofloxacin and florfenicol against ten (MIC data) or five (MBC data) koala isolates of *Chlamydia pecorum*.

Drug	MIC (µg/mL)			MBC (µg/mL)		
	Range	50%	90%	Range	50%	90%
Chloramphenicol	1 – 2	1	2	1 – 2	2	2
Enrofloxacin	0.25 – 0.5	0.5	0.5	0.5 – 1	0.5	1
Florfenicol	1 – 2	1	2	2	2	2

Chloramphenicol and florfenicol displayed comparable *in vitro* activity against *C. pecorum*; enrofloxacin displayed greater *in vitro* activity than either chloramphenicol or

florfenicol. On all eight testing days, the ofloxacin MIC for the reference strain was 1 µg/mL; the ofloxacin MBC for the reference strain was consistently 2 µg/mL.

5.5 DISCUSSION

This study provides novel information regarding the *in vitro* susceptibilities of commonly used antimicrobials against koala isolates of *C. pecorum*. Such information is valuable for the formulation of koala-specific drug dosages, and can also be used in conjunction with pharmacokinetic data to determine whether currently used dosage regimens are likely to achieve therapeutic targets *in vivo*.

There are currently no internationally accepted standardised guidelines for the antimicrobial susceptibility testing of *Chlamydia* spp. Despite this, the results of *in vitro* antimicrobial susceptibility testing for different chlamydial species using different cell lines are often similar (Bailey et al., 1984; Pudjiatmoko et al., 1998; Suchland et al., 2003). An exception to this is macrolide susceptibility when tested using different cell lines; *Chlamydia* cultured in BGMK cells yield higher MIC values for macrolides than those cultured in various other cell lines (Suchland et al., 2003). This was not the case for fluoroquinolones, and the results of this study indicate it is unlikely to be the case with chloramphenicol or florfenicol as susceptibilities to these drugs were similar to those previously published using other cell lines (Johnson and Hobson, 1977; How et al., 1985; Bianchi et al., 1988; Graham et al., 1988). BGMK cells were used in this study as they are a hardy cell line that is sensitive to chlamydial infection (Krech et al., 1989), producing large, easily-visualised inclusion bodies (Hobson et al., 1982b). Additionally, as an epithelial cell line, they serve as a good model for the epithelial host cells in which *Chlamydia* spp. grow and replicate *in vivo*. The reference strain used in

this study was tested against ofloxacin on each assay day, and results were consistently within the range of previously published MICs and MBCs for this drug when tested against *C. trachomatis* (Bailey et al., 1984; Suchland et al., 2003), reinforcing the validity of the method employed in this study.

The MIC_{TP} (Suchland et al., 2003) was used to determine MIC values in this study. This method has been adopted recently (Chu et al., 2010; Peuchant et al., 2011) to overcome the inherent subjectivity in determining an end point for chlamydial MIC determination with immunofluorescence staining. Using the MIC_{TP} method, visual inspection of serial antimicrobial dilutions reveals a noticeable change in inclusion number and morphology at the MIC_{TP} (Suchland et al., 2003) (Figure 5-1). This provides a method of endpoint detection that is likely to be consistent between operators. Using the MIC_{TP} to determine the MIC has produced MIC values within one dilution of those determined using molecular techniques (Peuchant et al., 2011), offering further support for this method of MIC determination.

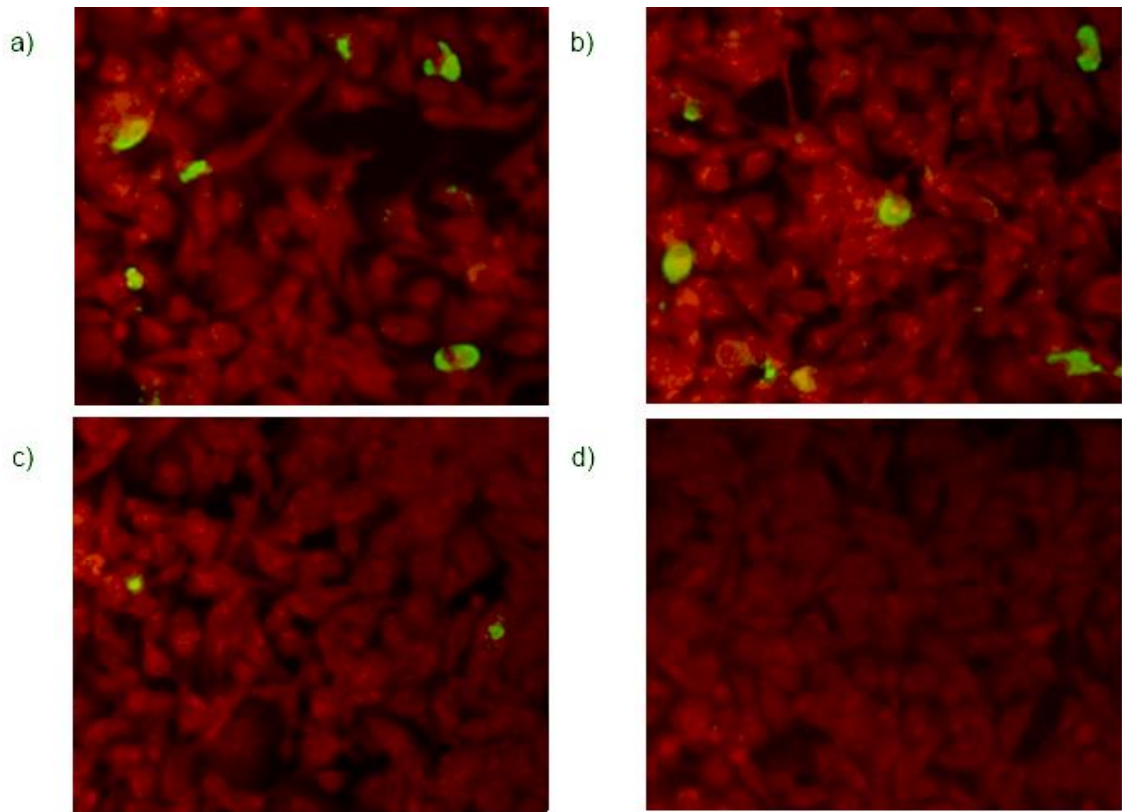


Figure 5-1. Sequential photographs of *C. pecorum* intracellular inclusions (green) in BGMK cells (red) following 48 h exposure to serial dilutions of chloramphenicol. a) 0.125 µg/mL chloramphenicol; normal Chlamydial inclusions. b) 0.25 µg/mL; normal Chlamydial inclusions. c) 0.5 µg/mL; few, small Chlamydial inclusions (MIC_{TP}). d) 1 µg/mL; no inclusions seen (MIC). The isolate was recovered from the left eye of a koala at the Currumbin Sanctuary Wildlife Hospital, Qld. Magnification, ×400.

Although chloramphenicol and florfenicol are not considered first line systemic antimicrobials for treating chlamydiosis in most species, they were investigated in this study alongside enrofloxacin. Chloramphenicol and enrofloxacin are routinely used systemically to treat koalas with chlamydiosis, as the macrolides and tetracyclines have been associated with a syndrome of inappetence, emaciation and death following administration to koalas (Brown et al., 1984; Osawa and Carrick, 1990). Although enrofloxacin is metabolised to an active metabolite, ciprofloxacin, in many species (Kaartinen et al., 1995; Mengozzi et al., 1996; Cester and Toutain, 1997; Kaartinen et al., 1997), the antimicrobial susceptibility of ciprofloxacin was not investigated in this study as enrofloxacin metabolism to ciprofloxacin is negligible in koalas (Griffith et al.,

2010), and we are unaware of anybody using ciprofloxacin to treat chlamydiosis in koalas. Florfenicol was investigated in this study as a potential alternative to chloramphenicol, as distribution of the commercially available chloramphenicol formulation recently ceased in Australia. The *in vitro* susceptibility of koala *C. pecorum* isolates to florfenicol and chloramphenicol were almost identical. Thus, based on *in vitro* susceptibility testing, florfenicol may be a rational alternative to chloramphenicol for treating koalas with chlamydiosis, and further investigation regarding the pharmacokinetics, efficacy, and safety of florfenicol in koalas with chlamydiosis is warranted.

The *C. pecorum* susceptibility to enrofloxacin in this study was the same as that of *C. pneumoniae* isolated from western barred bandicoots (MIC 0.25 – 0.5 µg/mL; Kumar et al., 2007); *C. psittaci* isolated from turkeys (MIC 0.25 µg/mL; Butaye et al., 1997); and to the susceptibility of ruminant isolates of *C. pecorum* to other fluoroquinolones (ofloxacin MIC 0.25 – 0.5 µg/mL, ciprofloxacin MIC 0.25 – 1.0 µg/mL; Pudjiatmoko et al., 1998). Additionally, *C. pecorum* susceptibility to chloramphenicol *in vitro* was similar to that of *C. trachomatis* isolated from humans [MIC 0.25 – 1.0 µg/mL (How et al., 1985); MIC 1.0 µg/mL (Johnson and Hobson, 1977); MIC 0.5 – 1.0 µg/mL (Bianchi et al., 1988)], despite the lack of a standardised testing procedure for chlamydial susceptibility testing. This supports the previous observation that chlamydial patterns of antimicrobial susceptibility display consistency across chlamydial species (Pudjiatmoko et al., 1998).

Up until now, pharmacodynamic targets for treating koalas with chlamydiosis have been based on the *in vitro* susceptibility of other chlamydial species to enrofloxacin and

chloramphenicol (Griffith et al., 2010; Black et al., 2012; Black et al., 2013), as *C. pecorum* susceptibility data for these antimicrobials were not available. The MIC values used to assess drug pharmacodynamics in these studies (1 – 4 µg/mL for chloramphenicol; 0.25 µg/mL for enrofloxacin) are in close alignment with the results of the current study, adding weight to the conclusions drawn in previous studies. Importantly, when combined with the available pharmacokinetic data (Griffith et al., 2010; Black et al., 2012; Black et al., 2013) and treatment efficacy data (Markey et al., 2007; Griffith, 2010; Govendir et al., 2012), the results of the current study offer further support for chloramphenicol as a suitable treatment option for chlamydiosis in koalas, as well as additional evidence that enrofloxacin is not a suitable treatment option for this disease at dosages likely to be safe in koalas. This claim is based on evidence of continued chlamydial shedding when enrofloxacin is withdrawn (Griffith, 2010), and a 0% probability of reaching therapeutic targets at the commonly used dose of 10 mg/kg subcutaneously every 24 h, when the lower MIC determined in this study, 0.25 µg/mL, is considered (Black et al., 2013). The enrofloxacin dosages calculated to be theoretically effective against *C. pecorum* are well above conventional dose rates, and bring with them concerns of toxicity (Black et al., 2013). Thus, until these dosages are determined to be safe, alternative treatment options should be explored.

This study provides *in vitro* susceptibility data for two antimicrobials commonly used to treat *C. pecorum* infections in koalas, as well as an antimicrobial that may hold promise for treating this disease in the future. Although *in vitro* susceptibility results do not always correlate with *in vivo* susceptibility, the data presented herein can be used as a guide for the determination of therapeutic targets during dose development. As well as highlighting similarities in the *in vitro* susceptibilities of different species of *Chlamydia*,

this study provides a rational justification for the investigation of florfenicol as a potential treatment option for this disease in koalas.

CHAPTER 6. GENERAL DISCUSSION AND DIRECTIONS FOR FUTURE RESEARCH

Prior to the commencement of this project, pharmacokinetic data for koalas were extremely limited, and there were no data available regarding the pharmacokinetics of any drug administered i.v. to koalas. The pharmacokinetic profile of a drug established following i.v. administration is the gold standard for pharmacokinetic studies as it enables estimation of absolute bioavailability (F), volume of distribution (V_d), and systemic clearance (CL); the latter is necessary for the determination of dosage regimens that will theoretically achieve therapeutic targets. Despite this paucity of pharmacokinetic data, many hundreds of koalas each year receive therapeutic drugs at a number of wildlife rehabilitation facilities across their natural range (Jones, 2008; Griffith et al., 2013), and there is anecdotal evidence that many of these drugs are effective at currently used dosages.

The work presented in Chapters 2, 3, and 4 describes the pharmacokinetics of three drugs that are commonly administered to koalas: chloramphenicol, enrofloxacin and fluconazole. The integration of pharmacokinetic and pharmacodynamic data for fluconazole, and for two formulations of chloramphenicol, as well as the use of population pharmacokinetic modelling and Monte Carlo simulation (MCS) for enrofloxacin, has enabled theoretical PK/PD assessments of whether the currently used dosages of these drugs are likely to be reaching therapeutic concentrations in koalas. Additionally, this work has raised further questions regarding the pathways involved in the metabolism of these drugs in koalas. The three drugs investigated undergo different metabolic and elimination pathways from one another in other species. Fluconazole is

primarily filtered by the kidneys and excreted unchanged in the urine (Humphrey et al., 1985; Brammer et al., 1991); the main route of enrofloxacin metabolism in many species is N-deethylation to an active metabolite, ciprofloxacin, via Phase I hepatic metabolism (Papich & Riviere, 2009b); and chloramphenicol is glucuronidated via Phase II hepatic conjugation into chloramphenicol glucuronide (Papich & Riviere, 2009a). By comparing the pharmacokinetic profiles of these drugs in koalas with those from other species, hypotheses can be formed regarding the rate and capacity of these metabolic and elimination pathways in koalas. Clinically, this information is invaluable for wildlife clinicians when making decisions regarding the choice of drug to administer to this species, and may hold similar implications for the treatment of other specialist folivores.

Prior to the work presented in this thesis, the limited data available regarding the pharmacokinetic profiles of various drugs in koalas led our research group to believe that many drugs administered to koalas were poorly absorbed after oral administration, and were eliminated at a rate so rapid as to render them inefficacious. This was based on preliminary studies of four drugs; enrofloxacin and marbofloxacin (Griffith et al., 2010), meloxicam (Kimble et al., 2013), and fluconazole (unpublished data). The work presented in Chapters 2 and 3 indicates that rapid elimination is certainly not the case for all drugs administered to koalas. Based on the CL and k_{el} estimated following i.v. chloramphenicol sodium succinate administration in Chapter 2, drugs that are metabolised by Phase II glucuronidation may be eliminated in koalas at a rate comparable to that seen in other species, if chloramphenicol is metabolised by glucuronidation in koalas as it is in other species. This hypothesis is further supported by previous evidence suggesting that specialist folivores, such as koalas, tend to rely

more heavily on extensive Phase I oxidation of their toxic diet, with Phase II conjugation reactions, including glucuronidation, being utilised to a lesser extent (McLean & Foley, 1997; Boyle et al., 1999; Lamb et al., 2004). Glucuronidation does occur to an appreciable extent in koalas for detoxification of ingested phenols, with glucuronides excreted in the urine at a rate approximately 1000-fold that of humans (McLean et al., 2003). However, the metabolic pathways involved in hepatic conjugation are saturable (Cassidy & Houston, 1984). Thus, it is possible that the net glucuronidation capacity available for drug metabolism in koalas is similar to what is seen in other, non-specialist species, if there is consistently some degree of saturation of the glucuronidation pathways involved in phenol metabolism. Terpenes are detoxified by Phase I metabolism in specialist feeders, even though these substrates are often glucuronidated by generalist feeders (Boyle et al., 2000b; Pass et al., 2001). The tendency of specialist feeders to metabolise these compounds by extensive oxidation, rather than conjugation, has been postulated as a method of conserving energy, and ensuring adequate conjugation pathways are available for the metabolism of phenols (Foley & Moore, 2005). Further work needs to be undertaken regarding the metabolic fate of chloramphenicol in koalas. However, if chloramphenicol is found to be metabolised primarily by Phase II glucuronidation in koalas, then the finding of comparable elimination rates between koalas and other species opens up a range of drugs that will potentially have a similar elimination rate in koalas to other species. It stands to reason that such drugs would be worthy candidates for pharmacokinetic and pharmacodynamic investigation in this species, as they are less likely to be subjected to the rapid metabolism experienced by some drugs that undergo Phase I oxidation reactions, such as meloxicam (Kimble et al., 2013), and are subsequently likely to be of clinical use in this species. Drugs that are utilised in koalas and undergo Phase II

glucuronidation in humans include the non-steroidal anti-inflammatory drug, carprofen (Ray & Wade, 1982); the opioids, codeine (Yue et al., 1991), morphine (Coffman et al., 1997) and buprenorphine (Chang & Moody, 2009); the analgesic, paracetamol (a.k.a acetaminophen) (Nelson & Morioka, 1963); and the general anaesthetic agent, propofol (Sneyd et al., 1994).

The finding of negligible metabolism of enrofloxacin to ciprofloxacin in Chapter 3 raises questions regarding the metabolic fate of this antimicrobial in koalas. Enrofloxacin is metabolised to ciprofloxacin in many species (Kaartinen et al., 1995; Mengozzi et al., 1996; Cester & Toutain, 1997; Kaartinen et al., 1997; Seguin et al., 2004) via N-deethylation (Papich & Riviere, 2009b), which is a Phase I hepatic microsomal oxidative reaction (Baggot & Giguère, 2013). However, alternative pathways for enrofloxacin metabolism have been found in rats, with both ciprofloxacin and a glucuronide metabolite being identified as major metabolites (Heitzman, 1995). Glucuronidation of enrofloxacin in koalas, if it occurs extensively, may be a reason for the lack of appreciable ciprofloxacin detection following enrofloxacin administration. This is purely speculative, and further work is required to identify the pathways responsible for enrofloxacin metabolism and elimination in koalas, as well as the nature of the metabolites produced. Such work was beyond the scope of this thesis, but would add to the growing field of knowledge surrounding koala metabolic processes.

In Chapter 4, fluconazole served as a useful standard for further assessing the differences in oral drug absorption (Griffith et al., 2010) and elimination (Kimble et al., 2013) that have been observed for some therapeutic drugs in koalas, when compared with other, non-folivorous species. Fluconazole is well known for having a uniform

pharmacokinetic profile across species, with almost complete oral bioavailability (Humphrey et al., 1985; Brammer et al., 1990; Craig et al., 1994; Latimer et al., 2001). The oral bioavailability documented in Chapter 4 was highly variable, and generally lower than that recorded for other species. This is in agreement with both of the studies that have investigated oral bioavailability of other drugs in koalas (Griffith et al., 2010; Kimble et al., 2013) and offers further evidence that this route of administration may be unsuitable for use in koalas. Further to this, Chapter 4 provided clear evidence for absorption rate-limited disposition of oral fluconazole in koalas, which has not been reported in any other species. It is yet to be determined whether the oral absorption of fluconazole was affected by the Infasoy paste with which the fluconazole was administered. However, in practice it is unlikely that oral fluconazole will be administered to koalas without use of such a vehicle, thus this concept is potentially a purely academic one.

Another convenient characteristic of fluconazole's uniform pharmacokinetic profile is that it enables allometric scaling across a wide range of species (Jezequel, 1994); this is not the case for many drugs (Riviere et al., 1997) and is presumably due to fluconazole's renal elimination pathway, as glomerular volume and number, effective renal plasma flow, and glomerular filtration rate (GFR) are parameters that can be scaled across species ranging in size from mice to whales (Edwards, 1975; Holt & Rhode, 1976; Singer, 2001). The finding of a poor correlation between koalas and other species when fluconazole parameters were scaled using allometry indicates differences in the disposition of this drug in koalas, particularly in relation to its elimination. This finding highlights the risks of using scaling techniques for species that have physiological characteristics that differ from conventionally studied species. The rapid

elimination of fluconazole also poses questions about whether this drug undergoes metabolism in koalas. It is possible that in koalas, as in many other species, the majority of fluconazole (70 – 90%) is excreted unchanged in the urine (Humphrey et al., 1985; Brammer et al., 1991), and that the rapid elimination rate seen in koalas is due to more efficient renal filtration of this drug. An alternative theory is that fluconazole may be subjected to metabolism in koalas. The amount of fluconazole excreted unchanged in the urine was not determined as part of the current project due to the difficulties associated with prolonged urine collection in koalas. However, should this be pursued in future studies, it may offer more information to explain the rapid elimination rate of fluconazole in this species by indicating how much fluconazole is renally excreted, and how much is eliminated by other mechanisms. Additionally, *in vitro* assays, such as hepatic or extra-hepatic microsome assays, would help to determine whether any appreciable metabolism of fluconazole occurs in koalas, and the nature of the metabolites produced.

The *in vitro* *C. pecorum* susceptibility data presented in Chapter 5 provided confirmation of the assumed susceptibilities of this pathogen that had been used for pharmacodynamic assessments of chloramphenicol and enrofloxacin in Chapters 2 and 3. These data helped to bridge the gaps in knowledge that were evident in the initial chloramphenicol and enrofloxacin pharmacokinetic studies undertaken in koalas (Griffith et al., 2010; Govendir et al., 2012), in which pharmacodynamic targets were assumed based on the published *in vitro* susceptibilities of other species of *Chlamydia* isolated from different host species, including humans. Standardised guidelines for antimicrobial susceptibility testing of *Chlamydia* spp. have not been developed, and the interpretation of *in vitro* antimicrobial susceptibility testing must be undertaken

cautiously, as *in vitro* results cannot be directly transferred to *in vivo* situations. However, the results presented in Chapter 5 helped to shed light on the likely suitability of each of these treatment options when combined with data from Chapters 2 and 3, and in light of previous data regarding response to treatment with these antimicrobials (Markey et al., 2007; Griffith, 2010; Govendir et al., 2012). The results of susceptibility testing of *C. pecorum* indicated that the MIC of chloramphenicol was at the lower end of the range used for the pharmacodynamic assessment of chloramphenicol in Chapter 2, and lower than that previously assumed for the *Chlamydia* spp.-chloramphenicol combination (Govendir et al., 2012). Consequently, the likelihood of reaching therapeutic targets against *Chlamydia* spp. when using chloramphenicol is potentially higher than that outlined in these studies. Based on all the available evidence, which includes the pharmacokinetic study presented in Chapter 2, the antimicrobial susceptibility study presented in Chapter 5, and a previous efficacy study (Markey et al., 2007), chloramphenicol appears to be a feasible treatment option for koalas with chlamydiosis. However, it is still questionable as to whether the current dosage of 60 mg/kg s.c. s.i.d. should be increased slightly. Importantly, the similarities in patterns of *in vitro* susceptibility between chloramphenicol and florfenicol, presented in Chapter 5 and determined previously (Graham et al., 1988), offer support for the potential of florfenicol in treating koalas with chlamydiosis if its safety can be demonstrated in this species, as chloramphenicol is no longer commercially available in Australia.

Due to the vast degree of between subject variability evident in the pharmacokinetic profiles of enrofloxacin in Chapter 3, population pharmacokinetic modelling was used for pharmacokinetic evaluation instead of the traditional non-compartmental analysis that had been used in Chapters 2 and 4. This had the added benefit of enabling MCS to

be performed. The use of probability theory enabled an estimation of the percentage of koalas likely to attain therapeutic targets when considering various enrofloxacin-pathogen combinations. This study provided overwhelming theoretical evidence that enrofloxacin is unsuitable for treating chlamydiosis in koalas. Prior to the study undertaken in Chapter 3, it was known that 10 mg/kg enrofloxacin s.c. s.i.d. was unsuccessful in effecting a microbial cure in koalas with chlamydiosis (Griffith, 2010). This was further supported by the *in vitro* susceptibility testing of enrofloxacin against koala isolates of *C. pecorum* presented in Chapter 5, which showed that the *in vitro* susceptibility of *C. pecorum* to enrofloxacin was the same as what had been assumed for the MCS. The doses of enrofloxacin estimated to achieve therapeutic targets against *C. pecorum* using the data from Chapter 3 were too high to warrant publication, as they carried concerns regarding sterilisation of the gastrointestinal microflora, and subsequent mortality of treated koalas. It is hoped that, as an outcome of this work, enrofloxacin will no longer be used to treat chlamydiosis in koalas, and that other treatment options will be sought.

As with any research, there were limitations encountered whilst undertaking the work presented in Chapters 2, 3 and 4. Some of these are common to the wildlife research setting, and include the limitations on the number of animals recruited for each study; the concerns associated with holding wild animals in captivity for prolonged periods, which precluded a cross-over design for the pharmacokinetic studies; and the remote location of the major field site relative to our laboratory.

Although many veterinary pharmacokinetic studies are based on sample sizes of six to ten animals, having higher numbers of animals in each group would have helped to

maximise the chances of recruiting animals that were truly representative of the sampled population. This was overcome to some degree in Chapter 3 with the use of population pharmacokinetic modelling, which enabled inter-individual variability to be incorporated into the analysis. However, this technique was not available for Chapters 2 and 4. The number of animals recruited for the pharmacokinetic studies were limited by both the number of wild animals available at the field site, and the number condoned on ethical grounds. As veterinarians, we are trained under the well-known injunction to ‘above all, do no harm.’ Thus, recruitment of healthy animals into a pharmacokinetic study is always going to be challenging, especially in the case of wild animals where the stress of human interaction has to be considered, and wherever possible, minimised.

As all of the koalas recruited into this study were wild animals that were being rehabilitated for re-release into the wild, their welfare was a major factor in the study design. When these studies were designed, the elimination half-life of these drugs in koalas was unknown, with the exception of the chloramphenicol base formulation (Govendir et al., 2012). It was anticipated that a washout period of at least one week would be required to enable a cross-over study to be performed. A washout period of a week, followed by a 24 – 48 h pharmacokinetic study and subsequent monitoring of the animals for another 48 h would have increased their time in captivity by a minimum of ten days. Not only can this cause undue stress to the animals, but it places extra strain on the resources of the wildlife hospital. This limitation could be overcome in the future by recruiting captive animals for use in pharmacokinetic investigations (eg. zoo display animals), although this requires ethics approval from the zoo’s governing body. Use of display animals would also require removal of these animals from display during the 24 - 48 h sampling period, in order to minimise concerns raised by the general public

caused by display animals being used for research as this often generates negative publicity.

As outlined in Chapter 2, the distance of the remote field site from the laboratory precluded quantification of the inactive prodrug, chloramphenicol SS, in the plasma following chloramphenicol SS administration, as this prodrug is only stable for up to one week in plasma when stored at -20°C (Nahata & Powell, 1981). This prevented determination of the true bioavailability of chloramphenicol following i.v. chloramphenicol SS administration, as the hydrolysis of chloramphenicol SS to biologically active chloramphenicol is known to be incomplete in those species studied (Glazko et al., 1977; Reiche et al., 1980; Koup et al., 1981; Burke et al., 1982). Whilst this limitation may have resulted in an overestimation of the CL and V_d in Chapter 2, this information is purely academic as the clinical use of chloramphenicol SS is limited in koalas due to its rapid CL, and the calculations performed regarding use of the absorption rate-limited chloramphenicol base formulation were not affected by this limitation.

6.1 POSSIBILITIES FOR FUTURE RESEARCH

6.1.1 Investigation of florfenicol as a treatment option for koalas with chlamydiosis

The work presented in this thesis provided theoretical evidence that the pharmacokinetic profile of chloramphenicol in koalas, combined with the *in vitro* susceptibility of koala isolates of *C. pecorum* to chloramphenicol, render chloramphenicol a suitable treatment option for koalas with chlamydiosis, although slightly higher doses may be recommended. This was in support of previous evidence that chloramphenicol, when

administered at 60 mg/kg s.c. s.i.d., successfully eradicated *Chlamydia* spp. from infected koalas (Markey et al., 2007; Govendir et al., 2012). Unfortunately, discontinued distribution of the commercially available chloramphenicol formulation in Australia was announced toward the completion of this project. As the work presented in this thesis indicated that enrofloxacin is not a suitable alternative to chloramphenicol for treating koalas with chlamydiosis (Chapter 3), an alternative treatment option is required. Although chloramphenicol can be compounded at some pharmacies, a compounded formulation has been tried, and there is anecdotal evidence that it has induced ulceration and abscessation at the injection site as well as diarrhoea and suspected gastrointestinal dysbiosis (A. Gillett, pers. comm.)³. However, the chloramphenicol derivative, florfenicol, has a spectrum of activity similar to that of chloramphenicol (Graham et al., 1988; Dowling, 2006), as well as a similar *in vitro* ability to inhibit the growth of *C. pecorum* (Chapter 5). Further work regarding the use of florfenicol to treat koalas with chlamydiosis requires initial tolerance studies to determine the safety of florfenicol in this species. Should adequate safety be demonstrated, follow-up pharmacokinetic studies and PK/PD integration or PK/PD modelling would be advantageous to enable dosage assessment and development. This could be followed up with efficacy studies to determine the effect of the established dosage of florfenicol on *C. pecorum* and *C. pneumoniae in vivo*.

³ Personal communication: Amber Gillett, Wildlife Veterinarian, Australia Zoo Wildlife Hospital, Qld, November 2013.

6.1.2 The use of *in vitro* models to determine the rate of metabolism of commonly used drugs in koalas

The use of *in vitro* models, such as microsome assays, to enable assessment of intrinsic CL and to identify metabolite production may provide valuable information regarding the metabolism of enrofloxacin and fluconazole, as the metabolism and elimination of these drugs seems different in koalas from other studied species. *In vitro* microsome assays could also be used to confirm whether chloramphenicol is metabolised by glucuronidation in koalas. Such *in vitro* assays may become an integral component of drug dosage development in this species in the future. Drugs that are anticipated to have favourable pharmacokinetic profiles in koalas, such as those that are metabolised by glucuronidation, could have their metabolic pathway and rate assessed prior to pharmacokinetic studies being undertaken, allowing an informed assessment as to the suitability of each drug for further development in koalas. This would reduce the number of pharmacokinetic studies undertaken using live koalas for drugs that are likely to be eliminated too rapidly to be of use in this species, and would enable more efficient use of time and resources.

6.1.3 Pharmacodynamic assessment of commonly used therapeutic drugs that are metabolised by glucuronidation

As the net glucuronidation capacity of koalas seems to be similar to that of many other species, assessment of the pharmacokinetic profiles of other drugs that are known to undergo glucuronidation in veterinary species and humans would be advisable. Many such drugs are used for analgesia, thus are widely used in wildlife hospitals where many cases are presented with traumatic injuries. Rational dosage development could follow pharmacokinetic studies of such drugs in koalas. By selecting drugs that undergo

glucuronidation, or at the very least by avoiding drugs that undergo Phase I oxidative metabolism by certain CYPs that are known to be highly active in koalas (Liapis et al., 2000; Ngo et al., 2000), the limited funding available for wildlife research could be targeted towards drugs that are likely to have favourable pharmacokinetic profiles in koalas.

6.1.4 Assessment of the pharmacokinetics of fluconazole when used in conjunction with amphotericin B

As fluconazole is often administered to koalas in conjunction with amphotericin B, it is prudent that further evaluation of this drug as a treatment option for cryptococcosis incorporates the concurrent administration of amphotericin B. Amphotericin B reduces renal blood flow and glomerular filtration rate (Burgess & Birchall, 1972; Tolins & Raij, 1988), which may slow the elimination of fluconazole. The subsequent increase in $AUC_{0-\infty}$ may be enough to allow theoretical pharmacodynamic target attainment when administering fluconazole at currently used dosages.

6.1.5 Investigation into the poor oral absorption of some drugs in koalas, and methods to enhance oral absorption of these drugs

Based on the limited rate and extent of oral fluconazole absorption in Chapter 4, and enrofloxacin and marbofloxacin previously (Griffith et al., 2010), there is scope for an investigation into the barriers impeding oral drug absorption in koalas. Whilst these barriers may in part be due to physical obstruction caused by the dense, finely masticated leaf matter that constantly fills the koala's stomach, there are other plausible explanations that warrant exploration, including drug chelation to divalent cations or feed constituents, efficient first pass metabolism, and the presence of efflux transporter

proteins (such as P-glycoprotein) that transport drugs back into the intestinal lumen following absorption into intestinal epithelial cells. If the factors limiting oral drug absorption in this species could be determined, it is possible that methods of disarming these barriers could be employed when oral drug administration is important, such as during fluconazole therapy for cryptococcosis. The cost of injectable fluconazole, combined with the long duration of therapy required for treatment of cryptococcosis, renders the oral administration route superior to the intravenous route. However, disarming the oral absorption barriers would need to be investigated with caution if such barriers have evolved to prevent koalas from succumbing to the toxic constituents of their *Eucalyptus* spp. diet.

6.2 CONCLUSION

This study investigated the pharmacokinetic profiles of chloramphenicol, enrofloxacin and fluconazole in koalas, and used the data generated to make pharmacodynamic assessments regarding the suitability of these drugs when used in koalas. Part of this work involved determining the *in vitro* antimicrobial susceptibility of koala isolates of *C. pecorum* to enrofloxacin, chloramphenicol, and the chloramphenicol derivative, florfenicol. The results presented in this thesis indicate that chloramphenicol has a favourable pharmacokinetic profile in koalas, and is potentially a feasible treatment option for chlamydiosis, although slight dosage adjustments may be recommended. Unfortunately, discontinued distribution of chloramphenicol base in Australia means alternative therapies must be sought, and the results of *in vitro* susceptibility testing indicate florfenicol may be an alternative worthy of investigation. In contrast to chloramphenicol, this study showed that enrofloxacin is not a suitable treatment option for chlamydiosis in koalas, and its use for this purpose should be discontinued.

Enrofloxacin may, however, be appropriate for use in various gram positive infections of koalas, and its use should be assessed on a case by case basis. Although this study found that fluconazole is inconsistently absorbed from the gastrointestinal tract and rapidly eliminated from the plasma of koalas, further assessment of its suitability in the treatment of cryptococcosis cannot be made until its pharmacokinetics are assessed in conjunction with amphotericin B administration. A positive and unexpected outcome of this study was the finding that both chloramphenicol and enrofloxacin are eliminated from koalas at rates comparable to other species, and are not subjected to the rapid metabolism and elimination seen with meloxicam (Kimble et al., 2013) and fluconazole (Chapter 4). This provides hope that other drugs may have pharmacokinetic profiles that favour their use in koalas, although further work regarding the metabolic pathways involved in the metabolism of these drugs in koalas is required to further assess the suitability of various drugs and drug classes for use in this species.

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APPENDIX I

BUFFER USED FOR HPLC

100 mM Phosphate buffer:

KH_2PO_4	6.40 g
K_2HPO_4	0.515g
MilliQ Water	500 mL
Adjust to pH 6.0	

APPENDIX II

CULTURE MEDIA CONSTITUENTS

Seeding Medium:

High glucose Dulbecco's Modified Eagle's Medium ⁴	435 mL
200 mM L-glutamine ⁵	5 mL
1M HEPES buffer ⁶	10 mL
Foetal bovine serum (not inactivated) ³	50 mL

Inoculation Medium:

High glucose Dulbecco's Modified Eagle's Medium ¹	435 mL
200 mM L-glutamine ²	5 mL
1M HEPES buffer ³	10 mL
Foetal bovine serum (inactivated; 56 °C / 30 min) ³	50 mL

Maintenance Medium:

High glucose Dulbecco's Modified Eagle's Medium ¹	435 mL
200 mM L-glutamine ²	5 mL
1M HEPES buffer ³	10 mL
Foetal bovine serum (inactivated; 56 °C / 30 min) ³	50 mL
Cycloheximide 1 mg/mL ²	0.5 mL

⁴ Hyclone: Thermo-Fisher Scientific, Scoresby, Victoria, Australia

⁵ Sigma-Aldrich, Castle Hill, NSW, Australia

⁶ In Vitro Technologies, Noble Park North, Vic, Australia.

2-SP (Chlamydial transport medium)

KH ₂ PO ₄	0.101 g
K ₂ HPO ₄	0.201 g
Sucrose (C ₁₂ H ₂₂ O ₁₁)	6.846 g
MilliQ Water	to 100 mL
pH adjusted to 7.2 – 7.4	
Filter sterilised with 0.2 µm filter	
gentamicin (40 mg/mL)	0.14 mL
vancomycin (50 mg/mL)	0.3 mL
amphotericin B (500 µg/mL)	6.25 mL

APPENDIX III

PROCEDURE FOR SHELL VIAL CULTURE OF *CHLAMYDIA PECORUM* AND *CHLAMYDIA* *TRACHOMATIS* USING BUFFALO GREEN MONKEY KIDNEY CELLS

Medium:

- High glucose Dulbecco's Modified Eagle's Medium (DMEM) with additives as outlined in Appendix II.
 - i) Seeding medium
Used for seeding flasks, shell vials and 96 well plates, and to medium change flasks 24 – 48 h prior to passaging.
 - ii) Inoculation medium
Used to medium change shell vial cultures (0.5 mL up to 2 h prior to inoculation of specimens) and 96 well plates (100 µL up to 2 h prior to inoculation of specimens).
 - iii) Maintenance medium
Medium change inoculated shell vial cultures (1 mL) or 96 well plates (200 µL) prior to 48 – 72 h inoculation.

NB: ALWAYS WARM MEDIUM TO 37°C PRIOR TO USE SO AS NOT TO SHOCK CELLS/BACTERIA

Foetal calf serum:*Heat inactivation*

- Thaw serum rapidly (in a heat block or clean water bath) and mix the contents of the bottle thoroughly once thawed.
- Divide into single-use aliquots.
- Heat a circulating water bath or heat block to 56°C.
- When the temperature reaches 56°C, place the thawed tubes in the water bath/heat block so each is completely immersed to the level of the serum, without immersing the cap.
- Time the process for 30 min. Agitate the bottles approximately every 5 min during the heat inactivation process to prevent gelling of the serum proteins and to promote more uniform heating of serum.
- After 30 min of heat inactivation at 56°C, remove tubes from the water bath and rapidly cool them in an ice bath. Prolonged treatment of serum at elevated temperatures will cause deterioration of serum components critical for growth of cells.

Determining BGMK cell density:

- Shell vials and 96 well plates require seeding of 3.0×10^5 cells/mL
- Dilute cells 1:1 in Trypan Blue, and count using a haemocytometer until three consistent densities are obtained.
- Count only living cells (clear) for the cell count, but count dead cells (blue) separately for a viability count.
- Once cell density is determined, aliquot into working dilutions for seeding shell vials, 96 well plates and maintenance flasks.

Setting up shell vial cultures:

- Recover BGMK cells from liquid N₂, and thaw rapidly.
- Prepare required volume of suspension, containing 3×10^5 cells/mL, by diluting ampoule of cells.
- Seed 0.5 mL of BGMK cell suspension per shell vial culture, or 100 µL per 96 well plate.
- To ensure even settling, shake tubes in the vertical plane before setting rack down on a horizontal surface for overnight incubation at 37°C in 5% CO₂ incubator.
- Before use, check each shell vial culture for correct pH and healthy monolayers that are 90% confluent
- Medium change cultures (replace seeding medium with 0.5 mL inoculation medium for shell vials, or 100 µL for 96 well plates, up to 2 h prior to inoculation of chlamydial specimens.)

Addition of *Chlamydia* spp.

- Rapidly thaw frozen vials of *Chlamydia* or frozen clinical swabs by agitating at 37°C (eg, in a water bath within class II biohazard safety cabinet). For clinical swabs stored in CTM, aseptically add sterile glass beads to the vials containing the swabs and seal. Vortex for 30 seconds.
- Inoculate *Chlamydia* spp. onto confluent monolayers at calculated inoculation density (aim for 100 µL diluted to yield $10^3 - 10^4$ IFU/monolayer) and replace lids on vials/plates. Seal with parafilm.
- Centrifuge shell vials at 2000 x g for 60 min at 36°C

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- Remove supernatant and add 1 mL maintenance medium to each vial (200 µL to wells in 96 well plates)
 - Incubate at 37°C in 5% CO₂ for 36 - 72 h in PC2 laboratory incubator.

Harvesting and immunofluorescence staining of shell vial culture coverslips:

- After incubation for 36 – 72 h at 37°C, decant maintenance medium into liquid waste disposal container (1% sodium hypochlorite).
- Rinse monolayer three times with PBS
- Drain tubes on paper towel saturated with 70% ethanol.
- Fix cultures in 1mL methanol for 10 min at room temperature, then decant and drain tubes on paper towel saturated with 70% ethanol.
- Carefully remove coverslips. Be gentle so as not to damage the cell monolayer
- Place 10-15 µL fluorescein-conjugated monoclonal antibody onto parafilm, and place coverslip onto this (monolayer-down), ensuring entire monolayer is covered with stain.
- Incubate in a humidified chamber as per manufacturer's instructions (30 min), ensuring stain does not dry out.
- Rinse in distilled water for 10 sec to remove excess antibody. Blot excess water and mount with mounting fluid (pH 9.4) when dried
- Examine through a fluorescence microscope. Scan the entire monolayer using minimum total magnification of 100x, and confirm inclusion morphology at 400x.
- EB and RB inclusions stain green against a red background
- Keep stained slides at 4°C for 3 days, or at -20°C for a few months

Quantifying *Chlamydia* spp. isolated from swabs:

- Change culture medium in shell vials (seeding medium to inoculation medium)
- Thaw a frozen Chlamydial aliquot and prepare four or five serial 10-fold dilutions in CTM (2-SP)
- Inoculate three confluent shell vial monolayers with 200 μ L of each dilution
- Centrifuge at 2000 x g for 1 h at 36°C
- Replace inoculation medium with maintenance medium
- Incubate in 5% CO₂ for 48 h at 37°C
- Fix and stain as outlined above
- Count the inclusions per cover slip at each dilution, and determine the dilution necessary to obtain 50 – 100 inclusions per coverslip monolayer. This dilution contains 50 to 100 IFU/0.2 mL

APPENDIX IV

SITE OF SAMPLE COLLECTION FOR CHLAMYDIAL SWABS USED IN SUSCEPTIBILITY TESTING, AND SIGNALMENT OF THE KOALAS SWABBED

Age (years)	Gender	Date swabbed	Site swabbed	Geographical location
5	Male	24/11/2012	Bladder wall	SE Qld
9	Male	2/1/2013	Conjunctiva	SE Qld
9	Male	16/1/2013	Cloaca	SE Qld
9	Male	19/1/2013	Conjunctiva	SE Qld
9	Female	21/1/2013	Bladder	SE Qld
9	Female	7/12/2012	Conjunctiva	SE Qld
11.5	Female	18/12/2012	Cloaca	SE Qld
1.5	Female	1990*	Conjunctiva	WS
6	Male	1990*	Penis	WS

***Denotes isolates cultured in 1990 and subsequently stored at -80 °C. SE Qld: South east Queensland. WS: Western Sydney.**

NB: Isolates from the top five koalas were used to determine MICs only. Isolates from the bottom four koalas, and the koala *C. pecorum* type strain (Marsh et al., 2011), were used to determine both MICs and MBCs.